American Journal of

Veterinary Research

CONTENTS

Wall Structure and Closing Mechanisms of the Bovine Teat 349
W. D. Pounden and James D. Grossman

The Relation of Feed to an Outbreak of Bovine Hyperkeratosis 355
Carl Olson, Jr., E. H. Cook, and E. M. Brouse

The Binding of Blood Serum *Gamma* Globulin by Sulfadiazine 366
I. Forest Huddleson, Evelyn Sanders, and Leonard A. Mattuno

Research on Foot-and-Mouth Disease. II. The Cultivation of the Virus in Explantations of Tongue Epithelium of Bovine Animals 371
H. S. Frenkel

Preliminary Studies on the Effect of Streptomycin and Other Agents on *Mycobacterium Paratuberculosis* 374
A. B. Larsen, T. H. Vardaman, and A. H. Groth

Toxicologic Studies of Aldrin (Compound 118) on Large Animals 378
C. H. Kitselman, P. A. Dahm, and A. R. Bergmann

Studies on Johnin. VIII. Preliminary Studies on the Specificities of Acid-Fast Allergens 382
L. A. Baldwin, B. W. Johnson, and A. B. Larsen

Experimental Therapy of Brucellosis in Swine with Streptomycin and Sulfadiazine 388
L. M. Hutchings, Doris E. Bunnell, and W. W. Bay

Studies on the Hematology of the Thoroughbred Horse. II. Weanlings 393
M. F. Hansen, A. C. Todd, C. W. Kelley, M. Cowein, and W. R. McGee

Studies on the Hematology of the Thoroughbred Horse. III. Stallions 397
M. F. Hansen, A. C. Todd, M. Cowein, and W. R. McGee

A Quantitative Investigation of Some Mineral Components of Wheat Plants 400
Margret Stuart, F. C. Harbaugh, and Joe Dennis

Studies of Canine Leptospirosis. I. Evaluation of Laboratory Diagnostic Procedures. II. Serologic Determination of the Incidence of Latent Infection in the Lansing, Michigan, Area 405
John P. Newman

A Study of Food Intake, Activity, and Digestive Efficiency in Different Type Dogs 412
W. T. James and C. M. McCay

The Prophylactic and Therapeutic Properties of Sulfamerazine in Fowl Cholera .. 414
J. O. Alberts

The Prophylactic and Therapeutic Properties of Sulfamerazine in Fowl Typhoid . 421
J. O. Alberts

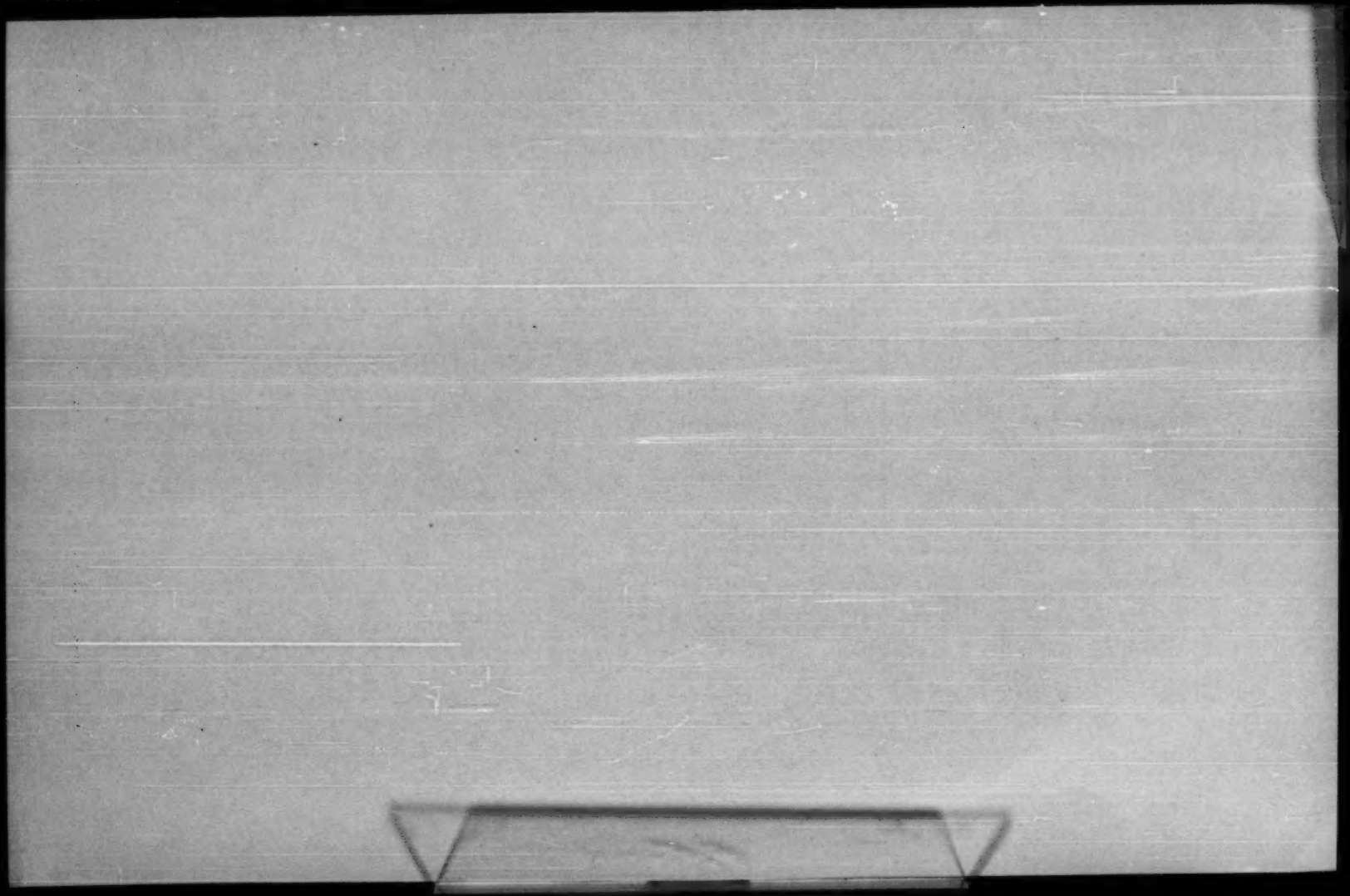
Search for Virus in Eggs Laid During Recovery Postinoculation with Newcastle Disease Virus 426
J. A. Bivins, Barbara Rhodes Miller, and F. R. Beaudette

Studies in Avian Leucosis. V. An Investigation of the Possible Relationship of Sex Hormones to Visceral Lymphomatosis 428
Olive Stull Davis, F. N. Andrews, and L. P. Doyle

Reinfection of Chickens Vaccinated by the Intranasal Method with Live B1 Newcastle Disease Virus 437
E. R. Doll, M. Elizabeth Wallace, and William H. McCollum

A Publication of the American Veterinary Medical Association

Volume XI | OCTOBER, 1950 | Number 41



Considering a new centrifuge? Look for these features . . .

- Compact, light in weight; fits in a small refrigerator when centrifuging in the cold is required.
- Provides a faster sedimentation rate and more complete sedimentation with finely dispersed particles through use of the angle principle—suspending the tubes at a fixed 52° angle.
- Can be used for micro and semi-micro work by substituting the proper shields and tubes.
- Maximum speed fully loaded is 4,000 RPM on AC, 4,500 RPM on DC.
- Rheostat has an "off" position and permits continuous speed control.
- Head is made of a special aluminum alloy, precision machined for accurate balance and protected by a hammertone finish.
- Cast aluminum housing is attractively finished in gray hammertone.

ADAMS SAFETY-HEAD CENTRIFUGE

Accommodates 6 15-ml. tubes. Complete with head, underguard, safety cap, shields and tubes; for 110 volts, AC-DC, CT-1002.....each $75.00

220 volt model, $3.00 additional.

Write for Circular 309VR describing our complete line of centrifuges

CLAY-ADAMS COMPANY, INC.

141 EAST 25th STREET · NEW YORK 10

Showrooms also at 388 West Washington Street, CHICAGO 6, ILL.

Editors

L. A. MERILLAT (editor emeritus), R. C. KLUSSENDORF, J. G. HARDENBERGH, HELEN S. BAYLESS. Committee on Journal: W. G. BROCK, W. M. COFFEE, and JOHN R. WELLS.

Research Council

Anatomy and Histology

L. E. ST. CLAIR, D.V.M., M.S., Ph.D. (*Secretary*), Professor of Veterinary Anatomy and Histology, College of Veterinary Medicine, University of Illinois, Urbana. (1952).

Bacteriology (Immunology and Biologic Therapy)

L. C. FERGUSON, D.V.M., Ph.D., Associate Professor of Bacteriology, College of Veterinary Medicine, The Ohio State University, Columbus. (1953).

Biochemistry and Animal Nutrition

T. J. JONES, D.V.M., School of Veterinary Medicine, University of Georgia, Athens, Ga. (1953).

Large Animal Medicine

JAMES FARQUHARSON, Division of Veterinary Medicine, Colorado A. & M. College, Fort Collins, Colo. (1953).

Large Animal Surgery

A. G. DANKS, B.S., D.V.M., New York State Veterinary College, Cornell University, Ithaca, N. Y.

Parasitology

W. E. SWALES, B.V.Sc., Ph.D., Officer in Charge, Branch Laboratory, Division of Animal Pathology, Dominion Department of Agriculture, Macdonald College P.O., P. Que. (1951).

Pathology

L. M. RODERICK, D.V.M., Ph.D., Head, Department of Pathology, School of Veterinary Medicine, Kansas State College, Manhattan. (1950).

Physiology and Pharmacology

D. K. DETWEILER, 48 Sproul Rd., Broomall, Pa. (1953).

Poultry Pathology

C. A. BRANDLY, D.V.M., M.S., Professor of Veterinary Medicine and Agricultural Bacteriology, University of Wisconsin, Madison. (1951).

Small Animal Medicine

C. P. ZEPP, SR., D.V.M., 136 West 53rd St., New York, N. Y. (1952).

Small Animal Surgery

C. F. SCHLOTTHAUER, D.V.M., Associate Professor, Division of Experimental Medicine, Mayo Foundation, Rochester, Minn. (1952).

Veterinary Hygiene

ANDREW L. MACNABB, V.S., D.V.M., D.V.Sc., Principal, Ontario Veterinary College, Guelph. (1952).

Virus Diseases

ALEXANDER ZEISSIG, D.V.M., Ph.D., Veterinary Consultant, New York State Department of Health, Albany, N. Y. (1952).

X-Ray

M. A. EMMERSON, D.V.M., M.S. (*Chairman*), Dr. Med. vet. (Zurich), Professor of Veterinary Obstetrics, Division of Veterinary Medicine, Iowa State College, Ames. (1951).

Member-at-Large

HADLEIGH MARSH, D.V.M., B.S., Veterinary Pathologist, Agricultural Experiment Station, Bozeman, Mont. (1951).

Published quarterly at 600 S. Michigan Ave., Chicago, Ill., by the American Veterinary Medical Association. Entered as Second-Class Matter January 31, 1941, at the Post Office at Chicago, Illinois, under the Act of March 3, 1879. Reproduction of any part of this publication is prohibited, unless special permission is given by the publishers and the authors. Subscription price: $4.00 per year for United States and countries affiliated with the Pan American Postal Union; Canada and foreign countries, $4.50. Members of the American Veterinary Medical Association may subscribe to the American Journal of Veterinary Research for $2.50 per year added to the annual dues of $10.00. Single copies $1.25. Address all correspondence to American Veterinary Medical Association.

ONE PRODUCT

To Prevent and Control Outbreaks of Cecal and Intestinal Coccidiosis

Poultry producers need your professional services for the competent diagnosis of *intestinal* coccidiosis and acute fowl cholera even though many believe that they can recognize *cecal* coccidiosis. Acquaint yourself with Sulfaquinoxaline, which offers the means of coping with these serious diseases in an effective, safe, and economical manner.

ACTIVITY: Sulfaquinoxaline is highly active against *Eimeria tenella, E. necatrix, E. acervulina, E. maxima, E. brunetti, E. meleagrimitis, E. meleagridis*—considered to be the most important species found in chickens and turkeys.

USES: Sulfaquinoxaline is the product recommended for *both* prevention and control of outbreaks of cecal and intestinal coccidiosis of chickens, and coccidiosis of turkeys. It enables birds to acquire immunity through mild infection. Sulfaquinoxaline also is used for checking immediate mortality in acute fowl cholera in chickens, turkeys, and pheasants.

ADMINISTRATION: Easily administered in the drinking water or in the feed—on a controlled dosage schedule.

MORE THAN 50 PUBLISHED REPORTS,[1] by outstanding poultry disease authorities, attest to the effectiveness of Sulfaquinoxaline.

[1] Annotated bibliography available on request.

UP-TO-DATE AUTHORITATIVE FACTS ON COCCIDIOSIS THERAPY

are summarized in this new booklet. It's yours for the asking. Please address requests to Merck & Co., Inc., Rahway, N.J.

Your veterinary pharmaceutical manufacturer supplies Sulfaquinoxaline in solutions, tablets, soluble powder, and premixes.

SULFAQUINOXALINE

MERCK & CO., Inc. *Manufacturing Chemists* RAHWAY, N. J.

DIFCO

PEPTONES AND HYDROLYSATES

for Microbiological Culture Media

Difco peptones and hydrolysates are prepared to meet the diversified nutritional and biochemical requirements of microorganisms. A single peptone cannot provide the essential nitrogenous nutriments for all cultural and metabolic processes. Difco peptones and hydrolysates have been developed to meet the various requirements for microbiological procedures including growth, preparation of toxin and vaccines, and for the study of microbial metabolism.

BACTO-PEPTONE is most widely used in the preparation of routine culture media. It is rich in readily available forms of nitrogen, and in a one per cent solution it is sparklingly clear with a reaction of pH 7.0. Bacto-Peptone is specified in the formulae recommended in "Standard Methods of Water Analysis" of the American Public Health Association.

BACTO-TRYPTOSE is a peptone which was originally developed for cultivation of the *Brucella*. A two per cent solution, as the sole source of nitrogen, is an excellent substitute for the meat infusion generally employed for propagation of the *Streptococci, Pneumococci, Meningococci* and other discriminative bacteria.

PROTEOSE PEPTONE is universally used in the preparation of media for elaboration of diphtheria toxin. It is also an excellent nutriment for use in media designed for the production of other bacterial toxins such as those of scarlet fever and botulinus.

PROTEOSE PEPTONE NO. 3 is particularly suitable for use in culture media employed for isolation and propagation of *Neisseria gonorrhoeae* and *Corynebacterium diphtheriae*

NEOPEPTONE is especially recommended as an ingredient of culture media for isolation and study of *Streptococci, Pneumococci* and Fungi. Media prepared with Neopeptone are most satisfactory for cultivation of *Streptococci* in the smooth or mucoid phase. Fungi produce typical characteristic colonies on solid media prepared with Neopeptone.

BACTO-TRYPTONE is especially adapted for the elaboration of Indol and for use in media to detect Hydrogen Sulfide production. This peptone is recommended in "Standard Methods for the Examination of Dairy Products" of the American Public Health Association for the medium used in milk counts.

BACTO-CASITONE is a pancreatic digest of casein recommended for use in the preparation of media for sterility testing according to standard procedures, and for the preparation of media requiring an enzymatic hydrolyzed casein. Bacto-Casitone can be used for the detection of Indol production by microorganisms, since it has a high tryptophane content.

BACTO-CASAMINO ACIDS is an acid hydrolyzed casein originally prepared for the production of diphtheria toxin in peptone free media, and since adapted for other toxins and vaccines. Iron, copper, and other heavy metals have been removed from Bacto-Casamino Acids. This hydrolysate is excellently suited for nutritional studies of microorganisms.

BACTO-CASAMINO ACIDS TECHNICAL is an acid hydrolyzed casein recommended for use in media where amino acids mixture are required as a source of nitrogen, and the sodium chloride content is not a limiting factor. Bacto-Casamino Acids Technical is of particular value in nutritional studies of bacteria.

BACTO-VITAMIN FREE CASAMINO ACIDS is acid hydrolyzed casein, free from vitamins. It is recommended for the preparation of media for microbiological assay of vitamins and tryptophane. This hydrolysate is a valuable aid in studies of growth requirements of microorganisms.

Specify "DIFCO"

DIFCO LABORATORIES

DETROIT 1, MICHIGAN

American Journal of Veterinary Research

COPYRIGHT 1950 BY AMERICAN VETERINARY MEDICAL ASSOCIATION

VOL. XI OCTOBER, 1950 No. 41

Wall Structure and Closing Mechanisms of the Bovine Teat

W. D. POUNDEN, D.V.M., Ph.D., AND JAMES D. GROSSMAN, G.Ph., D.V.M.

Wooster, Ohio, and Columbus, Ohio

FEW TISSUES of animal bodies are exposed to as much rigorous manipulation and risk of injury as the cow's teat. The development of protective methods against occupational injuries to this part of the mammary gland and improved methods of surgical repair of its tissues are dependent upon knowledge of its anatomy and the function of its various structures. This report concerns both structural and functional aspects of the teat wall and the outlet of the lactiferous duct.

THE TEAT WALL

The teat wall quite generally is regarded as being composed of three layers, a thin layer of skin closely adherent to an underlying muscular layer and a mucous membrane.[4, 7] The second layer of the teat wall —the muscular or intermediate layer—also contains bundles of collagenic and elastic fibers and many vessels.[7, 13] The superficial bundles of smooth muscle are largely longitudinal while, in the deeper portions, the bundles become more of the circular type.[7] The circular muscle is especially heavy about the outlet and forms a sphincter for the canal.[7] The muscle fibers are embedded in elastic and white, fibrous connective tissue.[5] The smooth muscle sphincter of the teat of the heifer appears in small bundles separated by considerable connective tissue as opposed to that in the mature, pregnant, and lactating cow, in which the circular muscle appears in large bundles and is separated by relatively little connective tissue.[13]

The mucosa, or third layer of the wall of the teat cavity, is composed of two layers of cells—tall columnar ones resting on quite low cells on a thin basement membrane.[13] A layer of connective tissue of varying thickness lies between the basement membrane and the intermediate layer and is referred to as the lamina propria.[7, 8] Immediately deep to the basement membrane, the structure is similar to that of the papillary layer of the skin.[8] It becomes more dense and contains bundles of collagenic fibers where it unites with the middle layer of the wall.[7]

The epithelium in the lactiferous outlet (streak canal, teat duct, papillary duct) differs from that in the teat cavity, being stratified squamous with the change occurring abruptly at the location of Fürstenberg's rosette.[13] Thick papillary and reticular layers form a heavy mass of connective tissue under and upon which the epithelial layer of the outlet lies.[7] A variable number of longitudinal folds, usually three or more, are present in the mucosa of the teat. Many of them begin as folds in the lactiferous outlet, continue as part of Fürstenberg's rosette, and extend through the cavity of the teat to join in the trabeculae in the lactiferous sinus.[7, 13] Transverse folds also are frequently visible, such as are pictured in an illustration by Sisson and Grossman.[11] Transverse folds or annular rings, which are located at the juncture of the teat cavity with the lactiferous sinus, are quite variable.[9] They sometimes are observed to be abnormally prominent, the incidence being

From the Ohio Agricultural Experiment Station, Wooster (Pounden); and the Department of Veterinary Anatomy, Ohio State University, Columbus (Grossman).

reported as 6.0 per cent by Rackow[10] and by Arnold[1] as 4.7 per cent. Pockets formed by the junction of transverse and longitudinal folds are present in 70 per cent of teats.[1]

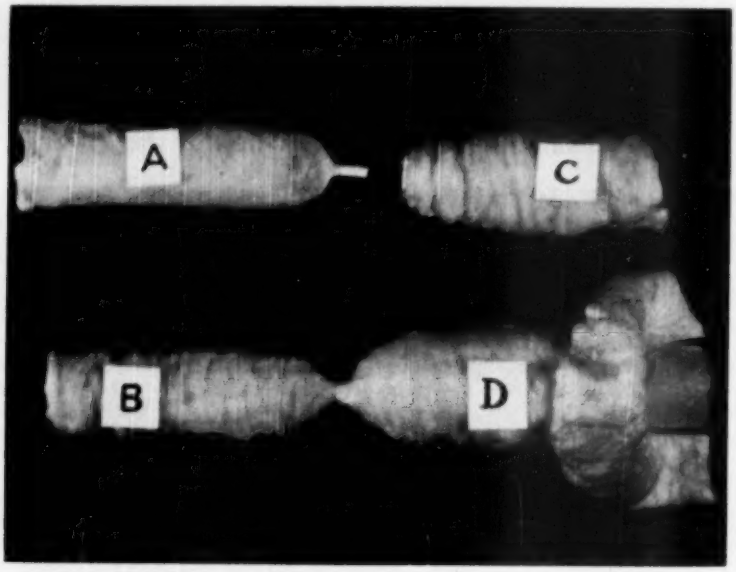


Fig. 1 a—Teat wall: (A) mucosa of teat cavity and outlet; (B) inner fibrous layer; (C) intermediate layer; and (D) skin and accompanying fibrous layer (inside out).

EXPERIMENTAL

Dissection of teats in the fresh state showed that a fibrous layer may be separated quite readily from between the mucosa and the intermediate layers. This was more than likely the connective tissue layer called the lamina propria of the mucosa and described as lying between the basement membrane of the mucosa and the intermediate layer.[7] The three layers, which are quite generally recognized along with this fourth, fibrous layer dissected out separately, are illustrated in the accompanying photograph and diagram (fig. 1 a and 1 b). This dissection shows the skin and its accompanying fibrous layer as a single piece (fig. 1 a, D) and joined to the inner fibrous layer (fig. 1 a, B). The figures illustrate that the corium of the skin and the inner fibrous layer were continuous with one another through the region of the lactiferous outlet. This might be expected inasmuch as, embryologically, the gland develops from invagination of the skin.[7] The intermediate layer (fig. 1 a, C), composed of smooth muscle bundles, connective tissue with numerous elastic fibers, and vessels, was soft and rubbery to the touch and dissected away from the adjacent layers in what apparently was a single layer. The mucosa (fig. 1 a, A) of the teat cavity joined the epithelium of the lactiferous outlet, and it, in turn, connected with the epithelium of the skin.

The epithelial lining of the lactiferous outlet and its supporting fibers were observed to be comparatively easy to free from the surrounding tissues, once a shallow circular incision was made around the external orifice of the teat. This ease of separation probably explains why these tissues are found sometimes extruding from the ends of teats which have been stepped on or crushed.

The fact that the fibrous layer and the mucosa can be readily separated would seem to indicate that the fibrous layer should be considered a separate layer and not merely a subsection of the mucosa. In further support of

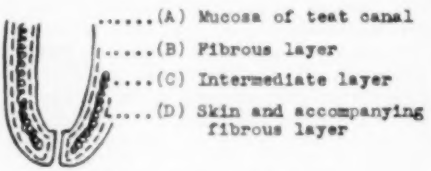


Fig. 1 b—Diagram of teat wall.

this, the fibrous layer seemed more adherent to the intermediate layer than the mucosa. It showed considerable tension, even more so than the other layers, and, consequently, may play a prominent role in maintaining the normal form of the teat. In surgical repair of penetrating wounds of the teat wall, it should be possible to keep fistulas to a minimum when the edges of the fibrous layer are brought into close apposition without any part of the mucosa protruding between them.

A number of teats were fixed in the distended position such as would exist when intramammary pressure was sufficient to cause milk to flow through them. This was accomplished by submerging them in 10.0 per cent formaldehyde and at the same time flowing similar solution through them under sufficient gravity flow pressure to maintain a constant stream through the lactiferous outlets. The teats were attached to pieces of tubing by tightly tying some of the adjoining udder skin and tissues around them with pieces of cord.

A point emphasized by this fixing technique was that the walls of teats elongated to a considerable degree beyond the normal resting position. Some increased almost an inch in length. At the same time, the walls, including the tissues around the lactiferous outlet, decreased in thickness (fig. 2) to about half that when in the resting position, a detail in agreement with the observations of others.[4,6]

This method of fixing caused the transverse folds, especially those at the proximal end of the teat cavity, to appear very prominent in some teats. Some of these large proximal folds were observed to be entirely separate from the glandular tissues. In other teats, these uppermost folds were absent or apparently were closely united with the distal parts of the glandular tissues. Submucosal veins frequently were more visible in teats fixed under pressure than in those fixed in resting states.

THE CLOSING MECHANISM OF THE OUTLET OF THE LACTIFEROUS DUCT

The smooth muscle in the vicinity of the outlet of the lactiferous duct is quite generally mentioned as being active in maintaining closure of the outlet.[5, 7, 11, 13] Fürstenberg's rosette provides a second mechanism for preventing leakage of milk because the loose folds press down against the duct opening.[5, 6] A thickening of the walls of the outlet, an overstimulation by the nerves of the muscle layers surrounding the outlet, or possibly the presence of large amounts of elastic connective tissue have been listed as possible causes of hard milkers.[5]

The smooth muscle bundles around the outlet are reported to be kept under constant tension by impulses from the autonomic nervous system.[5] Blocking of the inguinal nerve tended to temporarily relax the sphincter to some extent,[3] although sectioning of this nerve did not affect the normal "let-down" of milk.[2] Teats are capable of retaining milk even after the udder and all the nerves are excised.[9] This raises the question as to whether the nerves or the muscle bundles are as important in the functioning of the closing mechanism of the teat as generally regarded.

EXPERIMENTAL

Should muscle fibers under the control of nerve stimuli be of marked significance in maintaining closure of the outlet, then logically there should be a difference between the pressure required to open the outlets when a cow is alive and after slaughter. A limited number of trials were made in an effort to obtain data on this point.

Cows' udders were filled with milk through needles in the walls of the teats, or through milk tubes, until pressures were reached which would result in the teats leaking slightly. The pressures were recorded in millimeters of mercury and the experiment was repeated on the same udders after slaughter. When needles were used, the milk was forced in until the teats began to leak, at which time the pressure was recorded. When the milk was in-

Fig. 2—Inside of teat ends fixed under flowing pressure (larger) and in resting positions (smaller). Fürstenberg's rosette is formed by the light colored, prominent segments producing the ring surrounding the orifice in the center.

jected through a milk tube, sufficient milk was put in under pressure to cause the teat to leak on withdrawal of the tube. The pressure was recorded when it reached the point that the teat almost stopped leaking. In 1 case, the teats were severed from the udder after the recordings had been made and placed in the refrigerator for a month. They were then attached to glass tubes, were held upright, and the tubes filled until such time as the teats began to leak. The pressure necessary to cause them to leak was then recorded for comparison with the figures obtained previously.

Like the experiences of Espe and Cannon[4] using a sphygmomanometer on clamped-off teats, some difficulty was experienced in properly controlling all factors which might influence the results. For instance, needles passed through the walls of the teats sometimes caused hemorrhages inside the teats, which resulted in small clots, and occasionally these would obstruct the outlet. Then again, the repeated passage of milk tubes through the lactiferous outlet, even when done as carefully as was possible, tended to cause some variations. However, for the purpose for which they were used here, the measurements were considered to be reasonably satisfactory. Table 1 is a summary of the intramammary pressures which were required to cause slight leaking of milk through the teats of a few cows both before and after slaughter. Information is provided on the method of injecting the milk into the udder and the means used to enter the udder for making the recordings. Even though some differences were recorded between ante- and postmortem figures (table 1), they are sufficiently comparable to indicate that the closing mechanism of the teat must depend to quite an extent on factors other than nerve stimuli to the smooth muscle bundles. Undoubtedly, Fürstenberg's rosette plays a role in the operation of keeping the canal closed, as was discussed by others.[5,6] The ability of teats to retard flow through them following removal of the majority of their ends lends support to the belief that the rosette area functions in this respect. It has been suggested that ballooning of the teat on application of pressure flattens out Fürstenberg's rosette until it no longer interferes with the escape of milk.[7] For practical purposes, such apparently occurs, but it appeared from our study that the operation involved more than a flattening of the segments of the rosette alone, especially in teats having prominent rosettes. The location of the segments on the rim of the opening seemed to give them an ability to act as though elevated above the surrounding mucosa and thus able to form the lips of a valve. In order to open the lips, the segments were required to be pulled apart. This was accomplished, apparently, by the cordlike folds in the mucosa of the teat wall which continued dorsally to join in the trabeculae. These folds were more readily discernible in teats which were not distended. The walls of the teat cavity bulged outward when liquids were present under pressure and drew the cordlike folds with them. This pulled the segments of the rosette apart and produced a funnel for the liquids to pass through. This effect on the segments of the rosette was visible to some extent in the inside view of the ends of the teats which were fixed in the distended position (fig. 2).

Teats which had been severed from the udder and from which the rosettes were removed were still able to retain liquids. This indicated that other structures also were involved. The presence of elastic tissue fibers both in the upper and lower parts of teat walls

TABLE 1—Comparative Intramammary Pressures Required to Produce Slight Leaking by Teats of Cows at Ante- and Postmortem Examination

Cow	Teat	Pressure: mm. mercury		Method of injection and measuring
		Antemortem	Postmortem	
1	RR	30	28	Needle injected; tube measured.
1	LF	24	25	Teat tube injected and measured.
1	LR	31	27	Needle injected; tube measured.
2	RF	14	13	Teat tube injected and measured.
2	RR	22	22	Needle injected; tube measured.
2	LR	13	11	Needle injected; tube measured.
2	LF	18	14	Needle injected; tube measured.
3	RR	6	6	Needle injected and measured.
4	RR	20	11	Needle injected and measured.
5	RR	15	9, 13*	Teat tube injected and measured.
5	LR	11	12, 10*	Teat tube injected and measured.
5	RF	17	15, 12*	Teat tube injected and measured.

* = refrigerated thirty days.

has been mentioned by others.[8,12] They were noticeably plentiful in the region of lactiferous outlets. Those associated with the heavy mass of connective tissue upon which the lactiferous outlet epithelium rests tended to be in organ-

served still to be capable of retaining liquids to a comparatively marked degree. Consequently, it would seem rather likely that the elastic tissues play a significant part in maintaining closure of the lactiferous outlet.

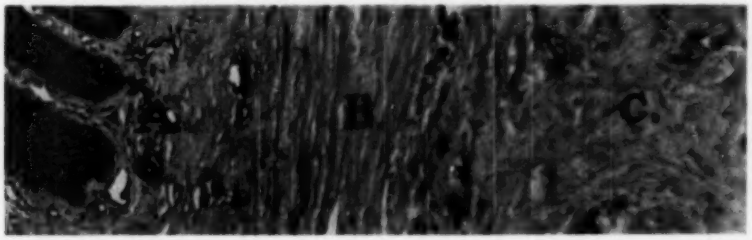


Fig. 3—Transverse section of teat in the region of the outlet of the lactiferous duct (x 100 and enlarged to approximately x 170): (A) epithelium basement membrane; (B) area of elastic fibers in circular formation around outlet; and (C) in scattered formation. Weigert's stain.

Fig. 4—Area of elastic fibers in circular formation around outlet. Similar to B in fig. 3 (x 440 and enlarged to approximately x 700). Weigert's stain.

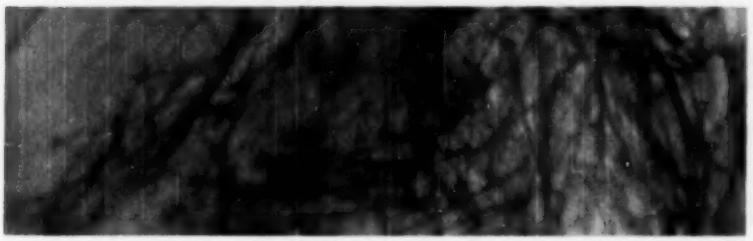


Fig. 5—Area of elastic fibers in scattered formation. Similar to C in fig. 3 (x 440 and enlarged to approximately x 700). Weigert's stain.

ized, straight bands (fig. 3 and 4), as though under tension in circular formation around the outlet. They presented quite a contrast to the more crisscrossed and scattered appearance of those located more laterally (fig. 3 and 5). The skin, muscles, and other tissues, with the exception of the epithelium and the supporting layers of connective tissues and associated elastic fibers, were cut away from around the ends of teats. The teats were ob-

Desquamated epithelial cells from the walls of lactiferous outlets formed waxlike masses in the outlet. Although this cellular débris was present in all teats examined, the greater quantities were in those from dry cows in which it was not unusual to observe quite firm plugs. When teats were cut open, these masses of cellular débris were noted to be rather easily rubbed off.

It was observed that lactiferous outlets,

from which most of the desquamated cells had been removed, frequently contained small amounts of milk between the longitudinal folds. On the other hand, only small traces or no milk at all remained between the folds in outlets in which the cellular débris was present in appreciable quantities. This seemed to be due to the fact that the masses of cells not only filled the area between the folds of the epithelium, but also had milk-repellent properties which prohibited milk from remaining between the surfaces of the masses. It appears, therefore, that these masses of cells may be another factor in maintaining closure of the lactiferous outlet, perhaps not so much against the outflow of milk as against penetration by foreign materials. The relative presence of masses of cellular débris in outlets conceivably may be a factor in the invasion of udders by mastitis infections. In the presence of milk residues, a pathway would be provided for growth of the organisms up through the outlets.

A comparison was made of the extent of the removal of the cellular débris from outlets by hand milking, machine milking, and by calf sucking, using severed teats attached to tubes in a manner to permit flowing liquids through them. The results indicated that hand milking removed the least and the machine the most, more especially when the action was prolonged for several minutes, including periods during which liquids were not flowing through the teats. In cases of teat erosion,[5,10] much of the desquamated epithelial mass probably would be removed.

It appears, therefore, that four structures are probably involved in maintaining closure of the outlet of the lactiferous duct:muscle bundles, Fürstenberg's rosette, elastic fibers, and desquamated epithelial cells. Indications are that Fürstenberg's rosette and elastic fibers may play roles at least as important as muscle bundles.

SUMMARY

Dissection of the wall of the teat of the cow indicated that the connective tissue layer lying between the intermediate layer and the mucosa probably should be considered as a separate layer and not a part of the mucosa. From a surgical standpoint, this means recognition of the following layers: the skin and the outer fibrous layer, the intermediate layer, the inner fibrous layer, and the mucosa.

Fixation of teats under sufficient internal pressure to simulate conditions of milk passing through the outlet of the lactiferous duct showed that the teat walls under such conditions became elongated and considerably thinner in comparison to resting states, an observation in agreement with the findings of others. Transverse folds sometimes became prominently visible, especially those at the proximal end of the teat cavity. In some teats, these folds were observed to be entirely separate from the glandular tissues, while in others, they were absent or closely united to them.

Rather similar recordings were obtained for the ante- and postmortem intramammary pressures which teats were able to retain without leaking. This was interpreted to indicate that muscle fibers under the control of nerve stimuli were unlikely to be responsible alone for maintaining closure of the outlet of the lactiferous duct. On the basis of the results of various examinations, four structures appeared to be involved in this operation: the muscle bundles, Fürstenberg's rosette, elastic fibers, and desquamated epithelial cells. Fürstenberg's rosettes and elastic fibers seemed to play roles at least as important as muscle bundles in milk retention. Milk remaining between the folds in outlets, and which could provide a pathway for growth of bacteria through teats, appeared to be least in outlets having relatively large quantities of desquamated epithelial cells present.

References

[1]Arnold, J. F.: Anatomy and Pathology of the Bovine Teat. J.A.V.M.A., *116*, (1950): 112-116.

[2]Ely, Fordyce, and Petersen, W. E.: Factors Involved in the Ejection of Milk. J. Dairy Sci., *24*, (1941): 211-223.

[3]Emmerson, M. A.: Some Observations on the Innervation of the Udder and Teats of the Cow and Their Relation to Milk Production. J.A.V.M.A., *74*, (1929): 372-375.

[4]Emmerson, M. A.: Studies of the Macroscopic Anatomy of the Bovine Udder and Teat. Pennsylvania Vet. Extension Quart., Bull. 41, (1940): 3-28.

[5]Espe, Dwight: Secretion of Milk. The Collegiate Press, Inc., Ames, Iowa, 3rd ed., 1946.

[6]Espe, Dwight, and Cannon, C. Y.: The Anatomy and Physiology of the Teat Sphincter. J. Dairy Sci., *25*, (1942): 155-160.

[7]Foust, H. L.: The Surgical Anatomy of the Teat of the Cow. J.A.V.M.A., *98*, (1941): 143-149.

[8]Little, R. B., and Plastridge, W. N.: Bovine Mastitis. McGraw-Hill Book Co., New York and London, 1946.

[9]Petersen, W. E.: The Action of the Mechanical Milker in Relation to Completeness of Milking and Udder Injury. J. Dairy Sci., *27*, (1944): 433-440.

[10]Rackow, H. G.: Bau und Entstehung der stenosierenden Ringfalten in der Zitze des Rindes. Deutshe tierärztl. Wchnschr., *49*, (1941): 569 (abstract).

[11]Sisson, S., and Grossman, J. D.: The Anatomy of the Domestic Animals. W. B. Sanders Co., Philadelphia and London, 3rd ed., 1938.

[12]Udall, D. H.: Teat Erosions. Cornell Vet., *37*, (1947): 73-77.

[13]Ventke, C. E.: Histological Study of the Teat and Gland Cistern of the Bovine Mammary Gland. J.A.V.M.A., *96*, (1940): 170-175.

The Relation of Feed to an Outbreak of Bovine Hyperkeratosis

CARL OLSON, JR., D.V.M., M.S., Ph.D., R. H. COOK, D.V.M., M.S., and E. M. BROUSE, B.Sc.

Lincoln, Nebraska, and Valentine, Nebraska

BOVINE HYPERKERATOSIS (hyperkeratosis or X disease) appears to be a distinct disease entity of bovine animals, according to the observations of many reporters. [12, 7, 10, 2, 4, 11, 1, 14, 15] So far, no conclusive evidence has been presented with respect to etiology of this disease. Among etiologic factors considered have been a transmissible infectious agent, nutritional components, and toxic substances. [13, 7] The disease has not been experimentally reproduced. Reports indicate bovine hyperkeratosis to be widespread in the United States.[7] Observations on the circumstances surrounding the various outbreaks have led to confusion rather than a rational pattern of factors involved.

An outbreak of bovine hyperkeratosis in a group of calves belonging to the Nebraska Agricultural Experiment Station afforded an unusual opportunity to study the disease. This occurred in an experimental group of calves arranged to test the efficiency of different types of rations for wintering of beef calves in dry lots. The observations suggest the association of certain nutritional factors with bovine hyperkeratosis and constitute the basis for this report. Also included are certain other observations related to the outbreak.

MATERIALS AND METHODS

History.—The Nebraska Agricultural Experiment Station maintains a substation located at Valentine, Cherry County, Neb. For more than twenty years, the animal husbandry department has conducted trials on the winter feeding rations of calves in dry lot somewhat similar to that begun in November, 1948.

On Nov. 4, 1948, 162 yearling Hereford calves were received at the substation for the feeding experiment. The calves were all raised on one ranch located about 50 miles from Valentine. Thirty heifers and 200 steers from the same calf crop were sold to another rancher in the vicinity. The 162 calves were fed prairie hay and a supplement of 1 lb. of oats and ¼ lb. of soybean-oil meal per calf until they were placed in lots and the feeding of test rations begun on Nov. 19, 1948. The calves were arranged in 16 lots of 10 calves each, leaving 2 extra calves. The feedstuffs for the calves consisted of prairie hay and various supplements. Two batches of prairie hay were fed, one harvested during 1948 and the other in 1947. The supplements were all prepared in advance and the various components were formed into pellets by a commercial feed company. In addition to the 160 calves in the feedlots, other cattle on the premises included 2 extra calves from the same source as those on test feeding, 6 Shorthorn cows, 1 Shorthorn bull, and 7 calves born from the milk cows (3 in 1948 and 4 in 1949).

The lots in which the calves were kept were arranged in two parallel rows running east and west. Each lot was approximately 26 by 100 ft. The adjacent lots were joined on the long dimension with a woven wire fence between them. Each pair of lots had a water tank along the fence, placed so that it would serve the cattle on both sides (fig. 1). No barn or shed space was provided. Along the north side of each set of lots was a high, solid fence to serve as a windbreak.

There was no sickness among the test calves until late in January, when emaciation was noted in 1 calf. The calf refused feed and a week later it had a sore mouth. The calf died Feb. 8, 1949. During the first week in February, 3 other calves were not eating well. These calves died in March. Veterinary service was not available in the vicinity of Valentine, and the record of the first part of the outbreak

Published with the approval of the director as paper No. 500 journal series, Nebraska Agricultural Experiment Station.

A contribution from the interregional project entitled "X Disease (Hyperkeratosis of Cattle)," a cooperative study participated in by the Agricultural Experiment Stations of the north central, northeastern, southern, and western regions, and the Bureau of Animal Industry, U. S. Department of Agriculture.

The authors gratefully acknowledge assistance from other members of the Department of Animal Pathology and Hygiene, University of Nebraska, Dr. E. P. Anderson, Nebraska state veterinarian, and Dr. J. E. Peterman, veterinarian in charge of Nebraska station, U. S. Bureau of Animal Industry and the veterinarians on their staffs who participated in this project.

From the Department of Animal Pathology and Hygiene, University of Nebraska, Lincoln (Olson and Cook); and the Valentine Experiment Substation, Valentine, Neb. (Brouse).

is sketchy. Early in March, tongues from 2 dead calves were submitted to the laboratory for examination. Papillomas* on these tongues aroused a suspicion of bovine hyperkeratosis. On March 16, the carcasses of 2 calves were brought to the laboratory for examination and a diagnosis of bovine hyperkeratosis was made. Five affected calves were brought to the laboratory for study on April 4. These had died by May 23. Plans were made to begin observations on the calves, but inclement weather following the severe blizzard of the winter prevented work until April 28, 1949. Lice were reported rather common on the calves in February. Specimens of lice were taken from representative calves in eight lots in June. Two species, *Bovicola bovis* (Linné) and *Haematopinus eurysternus* (Nitzsch) were found.**

The calves were sprayed with "rotenox" about April 23 and again sprayed for flies with DDT on July 6.

Rations.—The calves were fed prairie hay and various supplements. The supplements varied from lot to lot (table 4). The supplements included dehydrated alfalfa, dicalcium phosphate, soybean meal, cottonseed meal, urea, corn, and a carrier of vitamin A.†

The calves of lot 1 received only prairie hay of the 1948 crop. Prairie hay of the 1947 crop was fed to lots 14, 15, and 16. Calves in all of the other lots received prairie hay of the 1948 crop.

All of the calves had access to common salt in the form of the ordinary block salt for livestock. The drinking water came from the city of Valentine. The water was derived from a 160-ft. well driven through a stratum of red rock. A sample of this water was partially analyzed‡ with the following results:

Chloride (Cl)	4.00 p.p.m.¶
Nitrate (NO_3)	18.00 p.p.m.
Boron (B)	00.00 p.p.m.
Dissolved solids	264.00 p.p.m.
Selenium (Se) less than	0.01 p.p.m.
Fluoride (Fl)	0.10 p.p.m.
Specific conductance in microhms at 25 C.	334.00

¶ parts per million

The calves in lots 2, 5, and 6 began refusing to eat their pellet supplements during the latter part of February. During the first week of March, an attempt was made to feed the calves with a mixture of the pellets fed lots 3 and 15 (94% soybean meal and 6% dicalcium phosphate) and their regular pellet supplement. The calves refused to eat this. On March 1, the calves in lot 2 were given 1 lb. of soybean meal per calf per day. Lot 5 was given soybean meal, 1 lb. per calf per day and ground, shelled corn, 1 lb. per day per calf. The calves in lot 6 were each fed 1 lb. daily of the pellet designed for lots 3 and 15 (soybean meal 94% and dicalcium phosphate 6%). The supply of these pellets was exhausted about May 1 and from that time on, lots 6, 3, and 15 were fed soybean meal purchased locally. The calves in lots 2, 6, and 5 which were changed from the original ration of pellets took to their new rations rather slowly.

Early in May, the calves in lot 1 began chewing at the board fences indicating an aphosphorosis. They were then provided with about 1/10 lb. of steamed bone meal per calf daily. This seemed to satisfy their need for phosphorus and chewing on the fence stopped. Steamed bone meal was given at the rate of 1/10 lb. per calf per day to the calves in lots 5, 6, and 9 beginning May 26 and was given to the calves in lots 7, 11, and 12 beginning June 1. A new lot of prairie hay was fed to all of the calves beginning June 1.

On May 12, the calves in all of the lots were changed from their original ration and given 1 lb. of soybean meal per calf per day. One exception was with the group of calves in lot 1 that were continued on their ration of prairie hay alone. In other words, the original supplemental rations were not fed to any of the lots after May 12, and when the original supply of prairie hay was exhausted, a new lot of prairie hay (1948 crop) was fed to the calves after June 1. With the exception of lot 1, the supplemental ration fed all calves consisted of soybean meal, 1 lb., and steamed bone meal, 1/10 lb. per calf per day. Supplemental feeding of soybean meal to lot 1 was begun October 14. By this time, the calves were in rather poor physical condition and supplementary feeding seemed advisable to prepare them for winter.

In general, the calves were kept in their original lot arrangements until June 20, 1949. Several of the calves were moved to a hospital pen a few days before they died. By the end of May, all but 1 of the calves originally in lot 2 had died. In order to maintain contact with the disease for the calves occupying the adjacent lot 1, calves affected with the disease were moved from some of the other lots into lot 2. The population of sick calves in lot 2 was maintained at from 5 to 10 calves. On June 20, 1949, the entire group of calves, with the exception of lot 1, were rearranged in order to provide for some new experimental trials.

*The term proliferative stomatitis has also been applied to this lesion.

**The collection and identification were done by Dr. L. V. Skidmore.

†A commercial product containing 3,000 units of vitamin A per gram and manufactured from animal liver meal, fish liver oil, and soybean-oil meal.

‡This analysis was made by Dr. Herbert A. Swenson, chemist, Water Resources Division, Quality of Water Branch, Geological Survey, U. S. Department of Interior, Lincoln, Neb.

On August 15, a number of the calves which had been free of evidence of bovine hyperkeratosis for some time were permitted to go to pasture.

Procedure of Observations.—The plan for making observations on the outbreak of bovine hyperkeratosis consisted of leaving the calves in their original lots with no change of rations. Each of the calves in the test lots and the other cattle on the premises were individually examined at intervals. These examinations were made April 28, May 12 and 26, June 9 and 30, July 21, August 11, September 1, etc. Further examinations were made but for the purposes of this report only the first eight examinations will be considered. These cover approximately four months, during which the disease was at its peak and diminished.

The following specific symptoms, lesions, or abnormalities were looked for: (1) lacrimation; (2) emaciation (this was judged by the covering of fat over the ribs and was highly arbitrary); (3) salivation; (4) diarrhea; (5) skin lesions with a rough approximation of their location and extent; and (6) mouth lesions (particularly for the presence or absence of (a) papillomas in the oral cavity, (b) areas of ulceration or infection, (c) small, circumscribed red areas or erythema, (d) crescentic areas of punctate congestion in the mucosa, and (e) a condition called beginning papilloma (this consisted of a slightly raised, roughened area of the mucosa, not definite enough to be called a papilloma). Rectal temperatures of the calves were measured at the time of observations (103 F. or more was considered elevated). Other features under observation were abscesses and infections about the head, infections of the eye and cornea, the condition of the horns, etc.

RESULTS

The unusual circumstance of the outbreak occurring in a group of calves on experimental feeding trials permitted correlation of the disease with the feedstuffs given the different lots of calves. From an epizoötiologic standpoint, the data could be examined for evidence of contagion or transmission of the disease from lot to lot.

Mortality.—Death from bovine hyperkeratosis occurred in seven of the 16 lots of calves. The first calves died in February and mortality continued through November. The highest mortality occurred during the month of May, when 15 calves died. The mortality ranged from 10 per cent in lot 15 to 100 per cent in lots 2 and 9. Eight calves in lot 14 died. In lot 3, 5 calves died; lot 5, 5 calves died. In general, the deaths from any one lot were spread over a considerable period. For example, in lot 9, the deaths occurred during a period of eight months. Mortality in lot 5 occurred as follows: 1 in February, 2 in April, 1 in May, and 1 in August. Thus, these were spread over a period of seven months. The earliest severe death loss occurred in lot 6, in which 2 calves died in February, 4 in March, and 2 in April. The remaining 2 calves of lot 6 were both sick with the disease but were living at the end of November. Sixteen

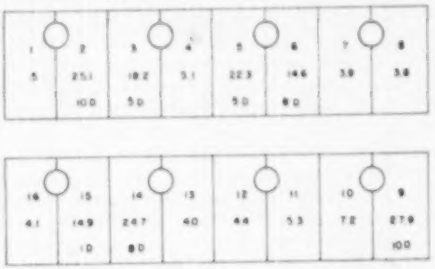


Fig. 1.—Arrangement of feeding lots to show the location of water tanks (circle) and severity of bovine hyperkeratosis in the different lots. Upper figure is lot number, second figure indicates rating value for the disease, and the third figure followed by D indicates the number of calves that died.

TABLE 1.—Classification of 142 Calves After Four Months of Bovine Hyperkeratosis Outbreak (April 28 to September 1, 1949)

	Lot															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Died	..	6	2	..	2	..	..	..	8	..	..	..	..	6	1	..
Sick	..	..	5	..	4	1	1	..	1	..	1	..	..	2	5	..
Sick and recovered	..	..	3	2	1	1	2	2	..	6	4	7	5	..	3	2
Suspect and recovered	..	..	..	5	..	..	3	4	..	4	2	..	3	..	..	4
Negative	10	..	..	2	..	..	3	3	..	..	3	3	2	..	..	4
Average ratings	0.5	25.1	14.2	5.1	22.3	14.6	3.9	3.8	27.9	7.2	5.3	4.4	4.0	24.7	14.9	4.1

TABLE 2—Frequency of Symptoms in Calves with Bovine Hyperkeratosis During Four Months of an Outbreak*

Symptom	Died		Continuously sick		Sick and recovered		Suspect and recovered		Negative		All calves	
	Observations (72)**	Calves (26)	Observations (168)	Calves (21)	Observations (320)	Calves (40)	Observations (200)	Calves (25)	Observations (240)	Calves (30)	Observations (1,000)	Calves (142)
Hyperkeratosis	98.6	100.0	95.2	100.0	35.0	100.0	26.0	92.0	0.0	0.0	40.5	77.5
Papilloma	76.8	100.0	45.8	85.7	14.0	40.1	0.0	0.0	0.0	0.0	17.3	49.3
Salivation	63.9	88.4	31.5	85.7	16.2	77.5	15.5	72.0	2.9	13.3	15.9	66.2
Emaciation	82.0	96.1	44.0	76.2	12.4	52.5	17.0	56.0	8.6	56.3	22.8	64.1
Lacrimation	82.0	92.3	64.3	100.0	52.1	95.0	59.0	100.0	26.6	46.6	51.6	85.8
Diarrhea	19.5	57.7	17.2	57.1	7.4	40.0	20.0	72.0	6.3	23.3	9.0	38.7
Ulceration	59.7	96.1	27.5	80.9	16.8	65.0	18.0	72.0	7.5	40.0	19.5	64.8
Begin. papilloma	26.4	46.2	26.2	80.9	25.9	80.0	24.0	76.0	14.5	56.6	22.8	68.3
Temp. 103 F.	33.2	69.2	9.5	61.9	7.8	45.0	6.5	40.0	6.2	26.6	9.2	47.2
Head infection	11.1	23.1	8.3	38.1	1.3	5.0	0.0	0.0	0.0	0.0	2.6	11.3
Crescent area	8.7	15.0	31.3	85.7	26.3	87.5	26.3	47.5	32.0	80.0	25.6	71.1
Red area	11.1	23.1	20.2	71.4	18.7	62.5	27.0	47.5	24.5	66.6	21.5	59.9
Warts	0.0	0.0	7.1	19.0	6.2	22.5	6.5	36.0	7.4	23.3	7.5	29.4

*Eight examinations on each calf unless it died. Expressed in percentage of examinations and percentage of number of calves in group.
**Number on which percentage was calculated.

calves, whose death was attributed to bovine hyperkeratosis, died prior to the beginning of organized observations on April 28. Of these 16, 7 were examined and diagnosed as bovine hyperkeratosis. It was assumed that the remaining 9, not examined by a veterinarian, died with the same disease, for the herdsman could ascribe no other reason. There were no deaths from bovine hyperkeratosis in lots 1, 4, 7, 8, 10, 11, 12, 13, and 16. Two calves died from a cause probably unrelated to bovine hyperkeratosis. One of these belonged to lot 15 and was observed by a veterinarian on April 11. The calf died "with tremors and convulsions similar to those seen in prussic acid poisoning; signs or evidence of the disease such as shown by other typical calves in the lots were not present." The other calf that died from an unrelated cause had been sprayed heavily with a benzene hexachloride solution four days prior to its death; no necropsy was performed on this calf. Both of these calves have been eliminated from consideration in the data.

Symptoms.—Since there were calves definitely affected with bovine hyperkeratosis in lots in which no mortality occurred, it became obvious that a study of the symptoms was necessary in order to establish any correlation between the extent of the disease in the different lots and feedstuffs given the calves. In the process of this analysis, a rating system was developed by which the relative significance of the different symptoms could be ascertained.

On the basis of eight observations made during a period of four months, the calves were divided into five groups. Twenty-six calves died with bovine hyperkeratosis and constitute one group. Two principal lesions of bovine hyperkeratosis (hyperkeratosis of the skin and papilloma of the mouth) were used as criteria for separating the remaining calves into four groups. Thirty calves remained negative and had shown neither hyperkeratosis of the skin nor oral papilloma during the period of observation. Twenty-one calves had either or both hyperkeratosis or oral papilloma at the end of the observation period and were regarded as calves continuously sick during this period. Forty calves had shown both hyperkeratosis and oral papilloma one or more times but were free of these lesions September 1 and were considered to be recovered cases of the disease. Twenty-five calves had

at no time shown oral papilloma but had at one or more times definite hyperkeratosis of the skin and were considered as suspects of the disease and recovered at the end of the observation period.

The data were examined to ascertain the severity of the disease in the different lots, using the five classifications (died with the disease, continuously sick, sick and recovered, suspect of the disease and recovered, and negative). These data are arranged in table 1. There were striking differences between the lots. For example, all of the 10 calves in lot 1 were negative for the disease, all 6 of the remaining calves in lot 2 died of the disease, and in the other lots the calves were distributed between these two extremes. In some, such as lot 3, 2 calves died, 5 were continuously sick, and 3 were definitely sick but recovered. In lot 4, 3 calves were definitely sick and recovered, 5 were suspect of the disease and recovered, and 2 remained negative. In those lots in which mortality occurred from the disease, there were no cases of calves suspect of the disease and recovered, or no negative calves (lots 2, 3, 5, 9, 14, and 15). A rather peculiar observation was that in each of lots 4, 7, 8, 11, 12, 13, and 16 from 2 to 4 calves were negative. This suggests that in those lots where the disease was relatively mild, some of the calves failed to show definite evidence of the disease.

The methods of comparing the lots related in the preceding paragraph left much to be desired. Consideration was not given to the other symptoms of bovine hyperkeratosis and neither did it give weight to the duration of these symptoms. It was reasoned that the frequency of the symptoms and lesions could be compared between the calves that were negative and the calves that were affected with the disease. The relative significance or importance of the symptoms would then be reflected in their difference of frequency between these two groups.

In table 2, the data are shown with respect to frequency of the various symptoms. This frequency was considered in two ways. One way was to base the frequency on the number of observations that were made on the calves of each group. For example, 21 calves were classified as being continuously sick during the four-month period of observation. During this time, eight observations were made on each calf, therefore, the frequency of the symptoms was based on 168 observations. The second method of estimating the frequency of symptoms was to base it on the number of calves in the group. By this procedure, a calf was considered as having shown the symptom if it were exhibited even once during the four-month period of the outbreak. It will be apparent that both methods of arriving at frequency of symptoms have merit and that they should be used in combination with each other.

Three symptoms or lesions were not observed at all among the group of negative calves. These were hyperkeratosis, papilloma of the mouth, and infection about the head. All of the other symptoms or lesions indicated were seen in the group of negative calves. Some of these symptoms, such as red crescent area of the mouth and red area of the mouth were seen with the same frequency in the negative group of calves as they were in the dead, sick, sick and recovered, and suspect and recovered calves. The condition of warts on the skin (common bovine papilloma) was probably an unrelated disease. The frequencies of these skin warts in the continuously sick, sick and recovered, suspect and recovered, and negative calves, were remarkably uniform (7.1%, 6.2%, 6.5%, and 7.4%, respectively), based on the number of observations. The percentage incidence of skin warts based on the number of calves in each group was likewise quite uniform (continuously sick, 19%; sick and recovered, 22.5%; suspect and recovered, 36%; and negative, 23.3%).

For the sake of simplicity, the data on all calves with bovine hyperkeratosis (including those dead, continuously sick, sick and recovered, and suspect and recovered) were grouped together and compared with data on the calves that were negative. The percentage incidence of symptoms based on number of calves only is given in table 3. The rating value given to a particular symptom or lesion was approximately the difference in percentage value between the affected calves and negative calves. Hyperkeratosis for example, occurred in 98 per cent of the calves affected with bovine hyperkeratosis and in none of the negative calves. Instead of using 100 as the rating, this was reduced to 10. Papilloma occurred in 63 per cent of the calves affected with bovine hyperkeratosis and in none of the

negative calves; its rating was therefore 6. Lacrimation occurred in 97 per cent of the calves with bovine hyperkeratosis and in 47 per cent of the negative calves; since the difference was 50 per cent, a rating of 5 was assigned to this symptom. Salivation occurred in 80 per cent of the calves with the disease and in 13 per cent of the negative calves. Here the difference in percentage was actually 67 but consideration was given to the percentage incidence in observations (*see* table 2) and the rating value was lowered to 5. Emaciation was observed in 68 per cent of the affected calves and 50 per cent of the negative calves. The difference being 18 per cent, a rating value of 2 was given to this symptom. Following this same scheme, diarrhea was given a rating of 2; ulceration, 2; beginning papilloma of the mouth, 1; elevated body temperature, 1; and infection about the head, 1. The lesion of red crescent area in the mouth occurred in 80 per cent of the affected calves and 69 per cent of the negative calves. It would thus appear that a rating value of 1 should be given to this lesion, however, examination of the percentage incidence based on observations, indicated no difference between the negative and affected calves. This lesion was therefore given a rating of 0. The same was true for the lesion of red area of the oral mucosa. In the case of skin warts (common bovine papilloma), the incidence was similar in the different groups of calves and no rating value was assigned to this condition. Oftentimes, some conditions such as hyperkeratosis, lacrimation, emaciation, and diarrhea were observed to be slight. In those instances, the rating value was reduced to half.

TABLE 3—Comparison of Percentage Incidence of Symptoms and Lesions of Bovine Hyperkeratosis in Sick and Negative Calves*

	Sick (112)**	Negative (30)	Rating
Hyperkeratosis	98	0	10
Papilloma	63	0	6
Lacrimation	97	47	5
Salivation	80	13	5
Emaciation	68	50	2
Diarrhea	43	23	2
Ulceration	71	40	2
Begin. papilloma	71	57	1
Temperature	52	27	1
Infection	14	0	1
Crescent area	80	69	0
Red area	67	58	0
Warts	23	20	0

*The rating values are based in part on the difference between these percentage values.
**Number of calves in group.

The average rating value for each calf was determined by using the ratings indicated in table 3. The rating for each calf at each observation was calculated and then the average for all eight observations was determined. The average rating for the calves that died was 27.2, with the lowest 20.0 and the highest 33.0. The rating for the group of calves that were sick was 18, with the lowest value 5.3 and the highest 27.9. The average rating for the calves that were sick and recovered was 7.1 (low 3.0 and high 12.4). For the group of calves suspect of the disease and recovered, the average rating value was 4.7, with a spread from 3.3 to 7.1. For the negative calves, the average rating value was 1.3, with a range of from 0.1 to 3.7.

One test that may be applied to such an artificial rating system to try its validity is to note the spread of the ratings in the various groups considered. There is a tendency for overlapping of the high and low values between groups. The greatest range of rating values occurred in the group of sick calves, where the values varied from 5.3 to 27.9. Such a wide range is probably to be expected since, from the group of sick calves, some will die and some will recover. Considering only the average values for the different groups, there was a reasonable difference between the five groups.

The average rating values were then determined for each of the sixteen lots of calves. These are shown in table 1 where

TABLE 4—Relation of Ration to Bovine Hyperkeratosis*

Lot	Alfalfa	Phosphate	Corn	Vit. A carrier	Soybean meal	Cottonseed meal	Urea	Rating
1	...	...	...	...	...	...	...	0.5
7	...	0.05	...	...	1.00	...	...	2.9
8	...	0.03	...	...	...	0.97	...	2.8
11	...	...	1.00	...	...	...	...	5.3
6	1.20	...	...	...	...	...	...	14.6**
12	...	...	0.88	...	...	...	0.12	4.4
13	...	0.08	0.81	...	...	...	0.11	4.0
2	...	0.06	...	...	0.94	...	...	18.2
15	...	0.06	...	...	0.94	...	...	14.9
4	...	0.05	...	0.01	0.94	...	...	5.1
16	...	0.05	...	0.01	0.94	...	...	4.1
3	1.95	0.05	...	...	...	...	...	25.1
14	1.95	0.05	...	...	...	...	...	24.7
5	1.12	0.08	...	...	...	...	...	22.3
9	0.82	0.08	...	...	...	...	0.09	27.9
10	0.46	0.08	0.36	...	...	...	0.10	7.2

*Test rations were fed calves from three to six months. Observations made during four months of outbreak, including last month of test feeding. Calves were fed prairie hay plus respective supplements. Figures represent daily ration in pounds.
**Based on 2 calves only.

they may be correlated with the classification of calves in the different lots. The average rating values varied from 0.5 in lot 1 to 27.9 in lot 9. There is, generally, good agreement between the rating system and the classification of the calves in the different lots. For example, in those lots where there was high mortality from the disease, there were high average rating values (lots 2, 5, 9, and 14). One exception is lot 6, in which a rating value of 14.6 was obtained from observations made on 2 calves which represented the lot during the time these observations were made. The rating value for lot 6 was obviously low, since the other 8 calves had previously died from the disease.

Relation of Ration to Bovine Hyperkeratosis.—There appeared to be a definite relation between the extent of bovine hyperkeratosis and the rations fed the calves (table 4). The rating system seemed to have particular significance in those lots which were given the same supplements (lots 3 and 15, 4 and 16, and 2 and 14). The calves in these pairs of lots received the same supplements but different prairie hay. That is, prairie hay from the season of 1948 was fed one lot and prairie hay a year older was fed the other lot. For lots 2 and 14, the rating values were 25.1 and 24.7. For lots 3 and 15, the ratings were 18.2 and 14.9. For lots 4 and 16, the ratings were 5.1 and 4.1 (table 4). In general, it seemed that the rating values were higher in those lots in which the calves received dehydrated alfalfa and dicalcium phosphate. The amount of dehydrated alfalfa received did not seem to have any marked effect for, in lots 2 and 14, the calves received approximately 1.95 lb. per day and in lot 9 the calves received only about 0.8 lb. per day. The rating values for these three lots were essentially similar (25.1 and 24.7, and 27.9, respectively).

A further examination was made of the constituents of the supplements to note their relation to bovine hyperkeratosis with respect to the rating for the different lots (table 4). Corn or soybean meal, when fed as the only supplement, seemed to have no marked effect on the bovine hyperkeratosis rating value. The rating values were high in lots 3 and 15, where dicalcium phosphate was added to the soybean meal. The same supplemental feeds of soybean meal and dicalcium phosphate with vitamin A carrier added, as in lots 4 and 16, were associated with lower rating values, suggesting a protective effect from the vitamin A carrier used. Dehydrated alfalfa fed alone as a supplement (lot 6) or fed with dicalcium phosphate was associated with a high bovine hyperkeratosis rating in lots 2, 14, 5, and 9. The low-rating value of 7.2 for lot 10, in which alfalfa, dicalcium phosphate, corn, and urea were fed, suggests that, in this instance, corn had a sparing or protective effect in the ration, for one would have expected the alfalfa and dicalcium phosphate to be associated with a higher rating for bovine hyperkeratosis. The rating value of 4.0 for lot 13 would suggest by indirection that corn had some protective effect against the dicalcium phosphate which this lot also received. The rating value of 3.8 for lot 8 suggests a similar protective effect of cottonseed meal. Urea seemed to have little effect on the ratings, for the value of 4.4 for lot 12 was not much different from that of 5.3 for lot 11. Urea was also a part of the ration for calves in lot 9, and the rating value for lot 9 was not much greater than that of lots 2, 14, or 5, lots which also received alfalfa and dicalcium phosphate but no urea.

An attempt was made to correlate the amino acid content (calculated from tables of analyses, Block and Mitchell[3]) of the rations with the severity of bovine hyperkeratosis. None could be recognized.

Epizoötiology.—The calves had been arranged and held in specified lots for about two months prior to the beginning of the outbreak and their arrangement was not materially changed during the first several months of the outbreak. The data could therefore be examined for evidence of contagion of bovine hyperkeratosis. No special precautions were taken to avoid carrying a hypothetic infection from one lot to another before or after the disease had become evident. When the calves were being examined during the course of the disease, the same chute was used for all calves in lots 1 to 8 and another chute was used for all calves in lots 9 to 16. No attempt was made to disinfect gloves or instruments between calves. A good opportunity existed at the time of observations for spread of infection from 1 calf to the next.

Since the greatest likelihood for contagion would have been among those lots having a water tank in common (fig. 1), these data will be examined first. Lots 1 and

2 were arranged in this manner. None of the calves in lot 1 were ever affected with bovine hyperkeratosis and their rating value was 0.5. All of the calves in lot 2 died with bovine hyperkeratosis and their average rating value was 25.1. As previously mentioned, other calves affected with bovine hyperkeratosis were moved from other lots into lot 2 in order to maintain exposure for calves in lot 1. Despite this, the calves in lot 1 remained negative for bovine hyperkeratosis. Four other pairs of lots, in which a water tank was common to each pair of lots, showed differences of the average rating value of the lots (fig. 1). These were lots 3 and 4, in which the calves had bovine hyperkeratosis rating values of 18.2 and 5.1, respectively. The calves in lots 9 and 10 had bovine hyperkeratosis rating values averaging 27.9 and 7.2, respectively. The rating values for bovine hyperkeratosis of calves in lots 13 and 14 were 4.0 and 24.7. The rating value for calves in lot 15 was 14.9, and 4.1 for the calves in lot 16. These observations on calves in lots where evidence of contagiousness should have been apparent rather definitely deny the possibility. Only two pairs of lots (7 and 8, and 11 and 12) had similar rating values in each of the pairs. In these instances, the rating values for bovine hyperkeratosis were low and the disease in the calves was rather mild. The eighth pair of lots (5 and 6) were probably about equal in the extent of bovine hyperkeratosis. The rating value of 14.6 for calves in lot 6 is obviously low, as previously mentioned.

TABLE 5—Constituents of Blood from Calves with Bovine Hyperkeratosis

	Calf 113*	Calf 118*	Calf 129**	Calf 13**	Normal***
Hemoglobin (Gm. %)	12.08	11.88	8.40 7.56 7.72	9.90 8.57 8.06	12.0
Glucose (mg. %)	103.00	90.00	116.00 67.00 78.00	90.00 60.00 47.00	40-60
Uric acid (mg. %)	3.37	2.98	3.11 1.10 2.06	3.04 1.82 2.24	0-2
Urea (mg. %)	54.50	6.50	11.00 5.50	7.50 10.75 8.75	6-27
N.P.N. (mg. %)	73.78	25.00	31.00 24.50 24.00	32.75 23.50 35.00	20-40
Total serum protein (Gm. %)	3.10	2.85	3.10 2.84 1.95	2.88 2.31 2.20	6.71
Serum albumin (Gm. %)	1.48	1.10	1.32 0.95 0.65	1.40 0.90 0.65	...
Albumin/ globin ratio	0.91	0.40	0.74 0.46 0.50	0.97 0.61 0.42	...
Inorganic phosphorus (mg. %)	10.20	5.65	6.45 5.80 5.90	6.41 6.62 6.20	3-10
Serum calcium (mg. %) (b)	12.75	13.60	14.25 12.4 12.2	13.2 12.8 12.1	...

*Observations on April 8, 1949.
**Observations on April 8, April 22, and May 3, 1949, respectively.
***Values from Dukes.[6]
All techniques from Technical Manual for Spectrochemistry, 1947, A. S. Aloe Co., St. Louis, Mo.; except calcium by the method of Clark, P., and Collip, J. P.: J. Biol. Chem., 63, (1925):63.

One feature suggesting contagion was the existence of disease in all but one lot of calves. Of other cattle on the premises, the 6 adult cows, 4 calves born in 1949, and a bull remained healthy. The 2 extra calves and the 3 calves born in 1948 from the Shorthorn cows did show some evidence of bovine hyperkeratosis. The latter 5 animals had some degree of contact with the calves in the lots for they were sometimes confined in runways adjacent to the lots. In addition, the 5 calves were sometimes fed the pellet supplements intended for the calves in the experimental feeding lots. The cows, calves born in 1949, and the bull were never in intimate contact with the calves in the lots nor were they ever fed the pelleted feed intended for the calves in the experimental lots.

When the nature and extent of the disease in the calves at Valentine became apparent, investigation was made of conditions at the ranch from which the calves originated. This investigation was made in April. There were over 400 cows on the ranch among which no unusual losses or disease problems had occurred. The calf crop subsequent to that furnishing the calves for the Valentine experiment was normal. The ranch that purchased the 230 calves of the same crop from which the Valentine calves originated was also visited in April, 1949. They were found in a healthy condition and the owner reported loss of only 3 calves during the unusually severe winter. These calves, subsequent to their purchase, were fed oats at first, then ground ear corn, dehydrated alfalfa pellets, a commercial protein supplement, and prairie hay. The owner believed he had unusually good results with the calves and the feeding regimé.

Biochemical Studies.—Two sets of spec-

trophotometric analyses* for vitamin A and carotene were made. The first set was on blood from a group of 4 calves (113, 118, 13, and 129) that died with the disease three, twelve, twenty-six, and forty-five days after the samples were taken. Values of 20 to 77 (ave. 42) μg. of carotene per 100 cc. of plasma and of 40 to 63 (ave. 50) μg. of vitamin A per 100 cc. of plasma were found. The liver of calf 113 had 2.4 μg. of carotene per 100 cc. of plasma and 5.4 μg. of vitamin A per 100 cc. of plasma. Less than 0.5 p.p.m. of selenium** was found in the liver of calf 113.

TABLE 6—Spectographic Analyses of Feed Given to Calves in Which There Was an Outbreak of Bovine Hyperkeratosis

	Cu	Mn	Co	Fe	Al	B	P	Na	Mg	K	Ca
Prairie hay (1948 crop)	6.4	108	0.10*	104	125	17	1500	184	1538	12230	6200
Pellets, lot 6** (alfalfa)	10.0	56	0.22*	130	150	52	3200	670	3500	27000	17000
Pellets, lots 2 & 14 (alfalfa + dicalcium phosphate)	8.4	45	...	320	760	46	3500	430	2200	24500	17000
Pellets, lot 5 (alfalfa + dicalcium phosphate)	7.4	44	...	350	790	46	8200	860	2400	27000	23000
Alfalfa hay reference material, standard sample	13.0	35	...	170	450	22	2500	940	3600	16400	18900
Pellets, lots 4 & 16 (soybean meal + dicalcium phosphate & vitamin A carrier)	19.0	37	...	230	110	40	14600	110	2800	27500	17800

*Determinations reported by K. C. Beeson, U. S. Soils, Plants, and Nutrition Laboratory, Ithaca, N. Y., by colorimetric method.
**Pellets, lot 6 additional analysis: 4.6 Mo, 24.0 Zn, and 4.0 lead.
Analyses by A. W. Specht, and reported by W. O. Robinson, Bureau of Plant Industry, Soils, and Agricultural Engineering, Washington, D. C. All results in parts per million

The second set of analyses was made on blood obtained May 26 from 1 or more representative calves from each of the sixteen lots. Vitamin A levels of the plasma from these 32 calves varied from 55 to 125 (ave. 80) μg. per 100 cc. of plasma. Carotene levels varied from 48 to 201 (ave. 126) μg. per 100 cc. of plasma.

All of these values were considered within normal limits and no reasonable correlation of high or low values could be made with the status of disease in the individual calves or the group of calves representing different experimental lots.

Some constituents of the blood of 4 diseased calves (13, 113, 118, and 129) were measured. The first samples of blood were obtained April 8 and second and third samples were obtained April 22 and May 3 on 2 of the calves. The values found showed no regular deviation from normal (table 5) that could be regarded as characteristic for bovine hyperkeratosis. The values for uric acid, urea, and nonprotein nitrogen in the blood of calf 113 may have been high because of terminal renal insufficiency.

Some analyses were made on feedstuffs fed to those lots of calves where the disease was severe. These data are incorporated in table 6. The pellets fed lot 9 were reported† to have DDT, 0.43 p.p.m. The values for lead, zinc, copper, cobalt, and DDT or TDE were regarded as essentially "normal," adequate, or within reasonable limits, and only the value for molybdenum was believed somewhat high by Robinson. No good correlation could be established between the values for elements in the feed and the severity of bovine hyperkeratosis in the groups of calves receiving the feed. For example, 14,600 p.p.m. of phosphorus was found in the supplement fed calves in lots 4 and 16, where the disease was relatively mild, and 3,500 p.p.m. of phosphorus was found in the supplemental feed for lots 2 and 14, where the disease was severe. This cannot be regarded as conclusive evidence because another factor of a vitamin A carrier was given to calves of lots 4 and 16. The data are presented as findings for the benefit of those who may wish to study them. Such study should give careful consideration to all of the factors and circumstances involved.

Observations on Cells of the Blood.—Repeated counts were made of erythrocytes and leucocytes in the blood of 4 calves that eventually died with bovine hyperkeratosis. Two of the calves showed a progressive anemia, confirming the observation of Huffman *et al.*[10] The values for heterophils (ave. 7,293; low, 1,300; high, 23,500/cmm.)

*These analyses were made by Dr. R. L. Borchers of the Department of Agricultural Chemistry, University of Nebraska.
**Analysis by Dr. A. L. Moxon, South Dakota Agricultural Experiment Station.

†Analysis reported by W. O. Robinson, Bureau of Plant Industry, Washington, D. C. Analysis for DDT by Dr. R. C. Roark, Bureau of Entomology and Plant Quarantine, Washington, D. C.

and lymphoctyes (ave. 8,284; low, 4,800; high, 11,500/cmm.) were generally higher than considered normal. The high values of heterophils were not surprising in view of the extensive infection found in the calves at necropsy. All had a pyogenic pneumonia as well as a necrotizing infection of the mouth. One calf had two subcutaneous abscesses on the jaw. The counts of eosinophils appeared to be lower than normal, but the number of observations was too small to establish such a conclusion clearly.

Therapeutic Trials.—Ten typically affected calves were selected for trials with the thought of allaying or preventing the secondary infections more than of attempting to cure bovine hyperkeratosis. Treatments were started May 26, 1949. Two calves (34 and 209) with average bovine hyperkeratosis scores of 25.5 and 17.0 were given penicillin at the rate of 300,000 units per day for five days. After a five-day interval, another five-day series was administered to each calf. This was repeated until four, five-day series of treatments had been given. No response was noticed. Calf 34 died Oct. 16, 1949, 108 days after the last dose of penicillin. Calf 209 recovered slowly from bovine hyperkeratosis but at no greater rate than other untreated calves. Two calves (N11 and 119), with respective average bovine hyperkeratosis scores of 17.1 and 22.0, were given sulfamerazine *per os* at the rate of 1 gr. per pound of body weight per day. The course of treatment was the same pattern as used for penicillin above. Calf 119 died twelve days after the treatment was stopped. Calf N11 slowly recovered from the disease after eight to ten months. Calves 52a and 77, with average scores of 28.0 and 10.9, received intravenously 10 cc. of sodium cacodylate for two successive days. The therapy was repeated in two weeks. No apparent beneficial results were realized. Calf 52a died twenty days after the last injection and calf 77 eventually recovered, although it was several weeks after the last dose of drug before signs of recovery were apparent. Calf 69, with an average score of 27.5, was given potassium iodide at the rate of 5 Gm. per day for five days. It died the sixth day. Two other calves, 3 and 52, with average respective scores of 26.6 and 14.2, were given potassium iodide *per os* at the rate of 5 Gm. per day for five days. The five-day series of treatment was repeated after a five-day rest period. Calf 3 died twelve days after the second series of treatments ended, while calf 52 very slowly showed evidence of recovery. However, the interval between treatment and recovery was so great that the therapy was given no credit. Calf N25, which had a score of 27.9, was given sulfamerazine at the rate of 1 gr. per pound of body weight for five days. Five days after the last dose of sulfamerazine, the five-day series of potassium iodide was started. Potassium iodide was given *per os* at the rate of 5 Gm. per day. No beneficial results were obtained from the treatment, although two months after the last dose, the calf began to recover.

Rectal temperatures were recorded before, during, and for some time after the courses of treatment in 9 of the 10 calves. No consistent change of the body temperature could be correlated with the therapy attempted.

Penicillin, sulfamerazine, potassium iodide, and sodium cacodylate, in usual therapeutic doses, had no effect on the course of the disease or secondary infections in the cases of bovine hyperkeratosis studied.

DISCUSSION

The results presented on the relation of feedstuff to bovine hyperkeratosis must not be regarded as conclusive. It must be recalled that the basic experiment was designed for a study of nutrition. The unusual coincidence of an outbreak of bovine hyperkeratosis in this experiment merely made it possible to study the relationship of the feedstuffs to the disease. The complexity of symptoms associated with bovine hyperkeratosis was a disturbing feature. Apparently, no single lesion or symptom can be regarded as specific for bovine hyperkeratosis. Certain lesions and symptoms regarded as associated with bovine hyperkeratosis can occur in cattle not affected with this disease. The approach for studying the outbreak of bovine hyperkeratosis involved the development of a rating system for evaluating the signs of the disease. Such a rating system is arbitrary and should be closely examined for defects of a serious nature. So far, it seems to meet the requirements of tests that can be applied. The rating system and the method followed in determining the relative importance of symptoms in bovine hyperkeratosis pertain to this particular study. At the present time, they need not be applied to clinical observations in the diagnosis of the disease.

The data from these observations suggest the existence of enhancing and protective factors. Certain elements in the feedstuffs appeared definitely to enhance bovine hyperkeratosis. These were dehydrated alfalfa and dicalcium phosphate. Other elements of feedstuffs, corn, the vitamin A carrier, and cottonseed meal seemed to have a sparing or protective effect against the factor or factors of dehydrated alfalfa and dicalcium phosphate. Information on the character or nature of these factors is purely speculative. The factors defined by these studies cannot be regarded as the cause of bovine hyperkeratosis. This is adequately demonstrated by the fact that the disease did occur in those groups of calves receiving supplements not containing dehydrated alfalfa or dicalcium phosphate. Analyses of the feedstuffs for trace elements or DDT-like compounds, have thus far failed to demonstrate abnormal levels of these substances that could be associated with severity of the disease. Previously, some conjectures had been made in this direction.[12] A careful study by Gibbons, Shoffeitt and Folse,[8] in which tissues from 6 cattle with bovine hyperkeratosis were specifically examined for DDT, has shown a lack of association of DDT with bovine hyperkeratosis.

The question of an avitaminosis A associated with bovine hyperkeratosis was raised by Olafson.[13] Huffman *et al.*[10] reported a low plasma-vitamin A level in a few instances of this disease. From our observations, the vitamin A and carotene levels of the blood and in the liver of cases of bovine hyperkeratosis were normal and adequate. The drinking water supplied the calves was of good quality and the selenium level low. The selenium content of the liver from 1 calf that died with the disease was low.

So far as could be determined, the inciting factor or factors responsible for the outbreak of disease seemed to exist only in the immediate area where the calves were kept. Other older and younger cattle on the premises, but not in the immediate vicinity of the feed area, were not affected with the disease. The cause of the disease must have developed after the calves were on the premises, for the remaining calves of the group from which these came remained healthy. The evidence indicating lack of contagiousness is in harmony with other reports and the many negative attempts to reproduce the disease (Olafson,[13] Gibbons,[9] Fox,[6] and others).

SUMMARY

An outbreak of bovine hyperkeratosis in a group of 160 calves fed various rations was studied. A rating system to evaluate the relative significance of the different symptoms and lesions of bovine hyperkeratosis was developed. The disease occurred in 15 of 16 groups of calves. The severity of the disease varied from lot to lot and, by use of the rating system, evidence was obtained to indicate an unknown factor or factors in the particular dehydrated alfalfa hay and dicalcium phosphate fed these calves that enhanced the severity of the disease. These factors may not be of primary etiologic significance, for the disease occurred in calves that did not receive dehydrated alfalfa hay or dicalcium phosphate. Some unknown substance or substances in corn, a vitamin A carrier, and cottonseed meal appeared to have a neutralizing or sparing effect on the severity of bovine hyperkeratosis.

The outbreak developed on the premises where the calves were fed, and there was no evidence to suggest that the disease was brought with the calves. There was no definite evidence of contagion associated with the outbreak.

References

[1]Allman, H. R.: Hyperkeratosis (X Disease). J.A.V.M.A., *114*, (1949) :29.

[2]Beck, J. D.: X Disease, Proliferative Stomatitis and Esophagitis, Hyperkeratosis. Vet. Extension Quart., *109*, (1948) :3-6.

[3]Block, R. F., and Mitchell, H. H.: The Correlation of the Amino Acid Composition of Proteins with Their Nutritive Value. Nutrition Abstr. & Rev., *16*, (1946-1947) :249-278.

[4]Boyd, C. L.: A Report on XX Disease in Texas. J.A.V.M.A., *113*, (1948) :463-464.

[5]Dukes, H. H.: The Physiology of Domestic Animals. Comstock Publishing Co., Inc., Ithaca, N. Y., 6th ed., 1947.

[6]Fox, F. H.: Personal communication, cited by Gibbons.[8]

[7]Gibbons, W. J., Lee, A. M., Johnson, A. G., and Robinson, W. O.: Report of the Preliminary Survey on "X" Disease (Hyperkeratosis) of Cattle. Part I. Mimeo. U. S. Dept. Agric., Feb., 1949.

[8]Gibbons, W. J., Shoffeitt, P. S., and Folse, D. S.: DDT and X Disease. J.A.V.M.A., *115*, (1949) :364-366.

[9]Gibbons, W. J.: X Disease of Cattle. Auburn Vet., *5*, (1949) :63 passim.

[10]Huffman, C. F., Duncan, C. W., Webster, H. D., Judd, A. W., Killham, B. J., and Thorp, F., Jr.: Hyperkeratosis (X Disease) of Cattle in Michigan. Michigan State Coll. Vet., *9*, (1949) :63 passim.

[11]Lawhon, G. J., Jr.: X Disease in South Carolina. North Am. Vet., *29*, (1948) :643-644.

[12]Conference on X Disease in Cattle, McCollum-Pratt Institute, Johns Hopkins University, Baltimore, Md., (1949) :1-13.

[13]Olafson, Peter: Hyperkeratosis (X Disease) of Cattle. Cornell Vet., *37*, (1947) :195-205.

[14]Simpson, Charles F.: Hyperkeratosis or X Disease in Florida. Vet. Med., *44*, (1949) :51-52.

[15]Udall, D. H.: Proliferative Stomatitis and Esophagitis (X Disease). "The Practice of Veterinary Medicine." Pub. by the author, Ithaca, N. Y., (1947) :732-733.

The Binding of Blood Serum *Gamma* Globulin by Sulfadiazine

I. FOREST HUDDLESON, D.V.M., Ph.D., EVELYN SANDERS, Ph.D., and LEONARD A. MATTANO, Ph.D.

East Lansing, Michigan

IN PREVIOUS investigations made to determine the binding of sulfonamides to proteins in blood plasma, it has been deduced from the experimental data that, under certain pH conditions, they are bound to the albumin fraction. Davis,[1] who has made extensive and careful investigations on this subject, appears to be the leading exponent of the albumin-binding hypothesis.

In the experimental data which follow, it will be shown that: (1) when a dilute mineral acid is added to a mixture of sodium sulfadiazine and undiluted bovine serum, a precipitate is formed which consists of sulfadiazine and a major portion of the γ-globulin fraction; (2) a solution of the fraction precipitated from a Brucella antiserum possesses bactericidal antibodies.

EXPERIMENTAL PROCEDURE

Although the soluble sodium salt of sulfadiazine was employed in the present study, other sulfonamides, such as sodium sulfathiazole, sodium sulfamerazine, and sodium sulfamethazine may be used for the same purpose.

The blood serums used were from Brucella-infected cows. The Brucella-agglutination titers of the serums varied from 1 : 1,000 to 1 : 20,000.

When a precipitation separation was to be made, the powdered salt of the drug was dissolved in undiluted serum at room temperature. The pH of the solution, during constant stirring, was lowered gradually to 7 by dropwise addition of 0.85 per cent H_3PO_4. Precipitation of sulfadiazine and serum globulin began immediately on addition of the acid.

Approximately, 80 per cent of the salt was precipitated at pH 7. The precipitate was collected on No. 50 Whatman paper in a Büchner funnel, washed thoroughly with 0.85 per cent NaCl solution, and then suspended in the same solution. While being stirred constantly, the insoluble material was dissolved by dropwise addition of 0.1 normal NaOH until a pH of 10 was reached. The solution was clear or slightly opalescent.

The concentration of sulfadiazine in any of the solutions studied was determined by the Bratton and Marshall method.[2] The amount of protein nitrogen in any given solution was determined by the semimicro-Kjeldahl method. The character and nature of the protein constituents separated from serum by sulfadiazine was determined electrophoretically in the modified Tiselius apparatus[3] by the Longsworth[4] scanning method after a 2 per cent protein solution had been equilibrated to a pH of 8.6 and ionic strength of 0.1 by dialysis against a barbiturate buffer.

The biologic activity (bacteriostatic or bactericidal) of the solution of sodium sulfadiazine and serum protein fraction, or the protein fraction alone, was determined by making serial twofold dilutions of a predetermined concentration of both in a liquid culture medium containing tryptose, glucose, thiamine hydrochloride, and sodium chloride. The complement-binding activity of the protein fraction with and without sulfadiazine was determined by adding 0.2 ml. of fresh, normal rabbit serum to each dilution to serve as complement. After the appropriate dilutions were made in 5 ml. of the culture medium, 10^5 live *Brucella abortus* cells were added to each dilution. The tubes were shaken and incubated at 37 C. for seventy-two hours. The presence of growth, as indicated by visible turbidity, was recorded at 24-hour intervals by minus or plus signs.

EXPERIMENTS AND RESULTS

Electrophoretic Analysis of Protein Constituents Separated from Blood Serum by Sulfadiazine Precipitation.—The procedure followed and results recorded in this particular experiment are similar to the many others that were made during this study. In order to obtain a large amount of the protein fractions, 20 Gm. of sodium sulfadiazine were dissolved in 100 ml. of serum from a Brucella-infected cow (991). Following the general procedure of precipitation and

Journal article 918, n.s., Michigan Agricultural Experiment Station, East Lansing.

From the Department of Bacteriology and Public Health, Michigan State College, East Lansing.

Dr. Mattano was formerly a fellow of the National Institutes of Health, Bethesda, Md.

resolution of the precipitate, sodium sulfadiazine was removed by a long period of dialysis against physiologic salt solution at 4 C. The final solution (400 ml.) was then concentrated to a protein content of 2 per cent by rapid evaporation in cellophane tubing exposed to a constant blast of air and equilibrated by dialysis to ionic

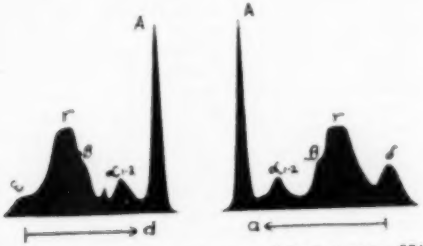


Fig. 1—Electrophoretic diagram of bovine serum 991 before addition of sodium sulfadiazine. Electrophoresis for 9,000 seconds at 5.86 volts per centimeter. Protein concentration 2 per cent.

strength and pH of the buffer solution previously described. A similar procedure was followed in the preparation of the supernatant solution recovered from the serum-sulfadiazine precipitate.

The electrophoretic diagrams of the serum before treatment, the supernatant from the serum-sulfadiazine precipitate, and the fraction removed with the sulfadiazine precipitate are illustrated in fig. 1, 2, and 3, respectively. Measurements of the

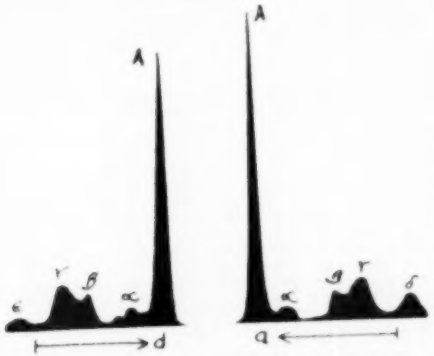


Fig. 2—Electrophoretic diagram of supernatant from bovine serum 991 after precipitation with sulfadiazine. Electrophoresis for 9,000 seconds at 5.90 volts per centimeter. Protein concentration 2 per cent.

area of the pattern components in fig. 1 revealed that approximately 48 per cent was in the region of the γ globulin. Approximately, 80 per cent of this component was removed from the serum by the sulfadiazine precipitate. This may be readily seen from the patterns (fig. 2 and 3) that developed from the supernatant and the redissolved precipitate. The shape of the large area in fig. 3 suggests that it is made up of three closely related constituents. Sufficient separation of the three has not been obtained to determine their character.

The electrophoretic diagram in fig. 3 shows the presence of a small component in addition to the large one; the mobility of the former was similar to that of albu-

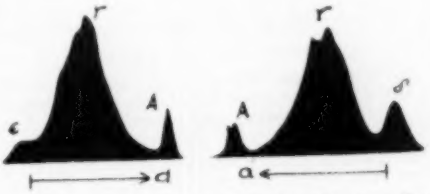


Fig. 3—Electrophoretic diagram of precipitate after removal from bovine serum 991 by sulfadiazine. Electrophoresis for 9,000 seconds at 6.18 volts per centimeter. Protein concentration 2 per cent.

min. This component separated into two distinct peaks in the ascending pattern.

The mobilities and relative compositions of the protein components shown in fig. 1, 2, and 3 are tabulated in table 1. It will be noted that there is close agreement in the calculated mobilities of the separate components in the serum before and after separation into two fractions. These figures are similar to those obtained in previous examinations of cow serum in this laboratory. A comparison of the relative compositions of the components in the serum before and after treatment with sulfadiazine shows very significant changes in their relation to each other. The serum γ/A ratio before treatment was 1.32. After removal of the sulfadiazine precipitate, it had changed to 0.35 (descending side) in the supernatant and 12.14 in the precipitable fraction. The differences in the ratios of β/A and α/A in the untreated and in the supernatant would indicate that a portion of α and β globulin was also removed in the precipitate.

Amount of Protein Nitrogen Removed from Serum by Sulfadiazine Precipitation.—The ratio of serum protein nitrogen removed from a Brucella antiserum to the amount of sodium sulfadiazine added is illustrated by the curve in fig. 4. In this experiment, varying amounts

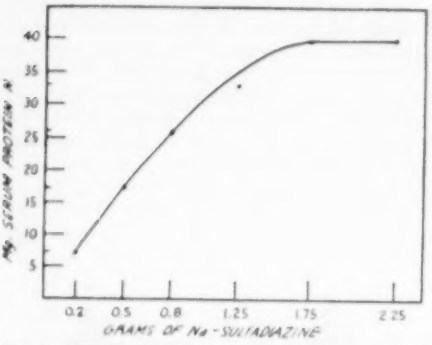


Fig. 4—Milligrams of serum protein nitrogen removed from 5 ml. of Brucella antiserum by varying amount of sulfadiazine during precipitation.

of sodium sulfadiazine were added to 5 ml. of the cow serum and precipitated by dropwise addition of 0.85 per cent H_3PO_4. The amount of protein nitrogen removed from the serum by each amount of sodium sulfadiazine was determined on the dissolved precipitate after the removal of all sodium sulfadiazine by dialysis. The protein nitrogen values recorded on the curve show that slightly more than 1.5 Gm. of the drug was required to remove the maximum amount (40 mg.) of protein nitrogen from 5 ml. of this particular Brucella antiserum.

Growth Inhibition Action of a Solution Containing Redissolved Serum Globulin and Sodium Sulfadiazine.—The results of numerous *in vitro* titration experiments show that the protein which the precipitated sulfadiazine removes from Brucella antiserums possesses complement-binding properties of a bactericidal nature. The *in vitro* activity of the antibody is more pronounced when a small amount of sodium sulfadiazine is added to the system. A similar increased activity has also been demonstrated in *in vivo* experiment.[6] The data shown in table 2 are an example of the results obtained in many similar experiments of this type. In this experiment, the blood serum used was from an infected cow (991) showing an agglutination titer of 1 : 20,000. The serum globulin was precipitated with sulfadiazine as previously described. The precipitate, after washing, was redissolved and filtered through a D8 Hormann sterilizing filter pad in a Seitz filter. The solution was found to contain 8 mg. per milliliter of sodium sulfadiazine and 0.33 mg. per milliliter of protein nitrogen. Two series of twofold dilutions were made of the filtered solution in 5-ml. amounts of the fortified tryptose medium in test tubes. The concentration of each agent in each dilution is indicated in table 2. To each dilution of one series, 0.2 ml. of fresh, normal rabbit serum was added as complement in order to measure Brucella antibody activity. Each dilution in both series was then inoculated with 10^5 *Br. abortus* cells. The tubes were shaken and incubated at 37 C. for seventy-two hours.

It will be noted from the results that the solution containing only sodium sulfadiazine and the serum-globulin fraction had only a temporary inhibiting effect on the growth of the organisms. The maximum inhibiting dilution was no higher than that of sodium sulfadiazine alone. The addition of complement to the dilutions of the serum fraction and sulfadiazine created a bactericidal complex which caused the death of the organisms in all dilutions except the last. The smallest amount of the serum fraction,

TABLE I—Mobilities and Relative Compositions of Protein Components in Bovine Serum 991 Before and After Separation into Two Fractions by Precipitation with Sulfadiazine

Figure No.	Volts per cm.	Side of cell	Mobilities u x 10^5					Relative composition				
			A	α_1	α_2	β	γ	A/G	α_1/A	α_2/A	β/A	γ/A
1 Serum[a] untreated	5.86	Descending	6.6	4.6	3.9	3.3	1.9	0.51	0.31	0.10	0.23	1.32
		Ascending	7.1	6.0		3.6	2.2	0.47	0.40		0.25	1.47
2 Serum, SD supernatant[a]	5.90	Descending	6.3	4.8	4.1	2.7	1.5	1.59	0.09	0.04	0.15	0.36
		Ascending	6.7	5.6	5.1	2.9	1.6	1.32	0.15		0.20	0.41
3 Serum, SD precipitate[a]	6.18	Descending	6.0				1.9	0.08				12.14
		Ascending	6.7				2.4	0.07				12.50

[a] Protein concentration 2 per cent; SD=sulfadiazine.

in the presence of the other two agents, which prevented the growth of Brucella organisms represented a protein nitrogen dilution of $1:2 \times 10^5$. It is possible that this fraction contains several specific antibody systems in addition to Brucella antibody. Therefore, it is not possible to make even a close estimate of the activity of the Brucella antibody in terms of the protein nitrogen which was separated from the serum by sodium sulfadiazine precipitation. Its maximum action will not become known until Brucella antibody protein can be separated from the fraction in a pure state.

Several *in vitro* titration experiments have been made to determine the activity of Brucella antibody in the protein fraction after the removal of sodium sulfadiazine by dialysis. The results have always shown that it is of a high order when estimated from the weight of protein nitrogen. In one experiment, typical of many, the protein nitrogen was diluted in serial twofold dilutions in the liquid culture medium. The concentration of protein nitrogen in the tubes ranged from 23 γ per milliliter to 0.0025 γ per milliliter. To each dilution, 0.2 ml. of fresh rabbit serum was added as complement, and 10^5 live *Br. abortus* cells. At the end of a 24-hour period of incubation at 37 C., there was visible growth in those tubes containing from 23 to 0.17 γ of protein nitrogen but none beyond this dilution to the final one. In other words, there occurred a zone of growth in the lower dilutions and a zone of growth inhibition in the higher dilutions of the fraction. Visible growth occurred in all dilutions at the end of a 72-hour period of incubation. It is possible that the zone of growth in the higher concentrations of the fraction was due to the presence of antibodies which were deficient in complement-binding activity. It has been shown in experiments presented in another paper[5] that inactive Brucella antibodies in a serum can be activated by the addition of sodium sulfadiazine and normal serum.

DISCUSSION

The nature of the reaction which takes place between sulfadiazine and the γ-globulin fraction of blood serum, when sulfadiazine is precipitated in blood serum by dilute acid, is still being investigated. The physical character of the precipitate which forms indicates that the reaction is not one of co-precipitation or adsorption. On the other hand, the fact that sodium sulfadiazine can be removed from the redissolved precipitate after a long period of dialysis would indicate that the binding or chemical reaction between the two agents is a loose one.

The fact that a specific and highly active complement-binding antibody for Brucella organisms was demonstrated in the protein which precipitated with sulfadiazine clearly demonstrates that the drug, under certain conditions, has a selective affinity for protein molecules in γ globulin possessing the property of antibodies.

TABLE 2—Bactericidal Action of Na-Sulfadiazine and Globulin Fraction After Precipitation and Resolution from Antiserum 991

Complement added to each tube (a)	Concentration of agents in tubes, γ/ml.								Bacteria control 5 ml. of medium
	NaSD →	80	40	20	10	5	2.5	1.25	0.62	
	Protein N. →	3.3	1.5	0.8	0.4	0.2	0.1	0.05	0.025	
	Inc. period hours	Degree of growth, turbidity								
0.2 ml.	24	— →							1+	2+(b)
	72	— →							2+	6+
None	24	— →					1+	2+	2+	2+(b)
	72	4+	4+	4+	4+	4+	4+	6+	6+	6+

10^8 *Brucella abortus* added to each dilution and controls.
(a) Fresh, normal rabbit serum.
(b) 0.2 ml. of fresh, normal rabbit serum added to control tube.
— = no visible growth; + = degree of growth.

The shape of the large component shown in fig. 3 would indicate that there are three closely related components in the area. They have not yet been separated for biologic or chemical studies.

SUMMARY

It has been shown that the addition of phosphoric acid (H_3PO_4) to a mixture of sodium sulfadiazine and bovine serum produces a precipitate which consists of serum protein and sulfadiazine. The physical character of the protein removed by the drug was determined by electrophoretic analysis. Most of the protein removed had the characteristics of γ globulin.

The protein fraction separated from Brucella antiserum by means of sulfadiazine precipitation has been shown to have antibody properties of a high order. The antibody in the presence of serum complement will inhibit the growth of Brucella organisms. These two agents in the presence of a low concentration of sodium sulfadiazine form a bactericidal complex.

References

[1]Davis, B. D.: The Binding of Sulfonamide Drugs by Plasma Proteins. J. Clin. Investigation, *22*, (1943):753-762.

[2]Bratton, A. C., and Marshall, E. K., Jr.: A New Coupling Component for Sulfanilamide Determination. J. Biol. Chem., *128*, (1939):537.

[3]Tiselius, A.: A New Apparatus for Electrophoretic Analysis of Colloidal Mixtures. Trans. Faraday Soc., *33*, (1937):524-531.

[4]Longsworth, L. G.: The Observation of Electrophoretic Boundaries. Ann. New York Acad. Sci., *39*, (1939):187-202.

[5]Huddleson, I. Forrest: The Potentiating Action of Sulfonamides on the Brucella Antibody-Complement System. Am. J. Vet. Res., *9*, (1948):277-285.

Research on Foot-and-Mouth Disease. II. The Cultivation of the Virus in Explantations of Tongue Epithelium of Bovine Animals

H. S. FRENKEL

Amsterdam, Holland

USING TECHNIQUES described in previous papers,[1,2,3,4,5,6,7] we have succeeded in cultivating the virus of foot-and-mouth disease in tissue cultures of embryonic bovine, ovine, and porcine skin. Cultivation was successful in Petri dishes and Roux and Kolle flasks. The important difference between the method reported in this paper and other published methods[8,9,10] is that the virus propagated by the technique described here is capable of producing the typical disease and lends itself to vaccine production and the preparation of hyperimmune serum. With the previously described methods utilizing embryonic tissue, the quantity of epithelium collected was rather limited. It was not considered practical to undertake extensive virus cultivation with such tissues collected at random from fetuses in the abattoir. The virus so produced was of considerable infectivity, but its concentration in the tissue was not regularly high enough for practical use in vaccine production. Since the epithelial cells of the normal bovine tongue are known to be especially sensitive to the virus, experiments were undertaken with such tissue.

Histologic examination of the aphthous vesicles has revealed that the lesions ordinarily begin in the deep layers of the skin, particularly in the stratum spinosum, and that, in the beginning of the vesicle formation, the cells of the stratum germinativum do not contain microscopic lesions.[11] With this information available, and in consideration of the fact that the outer layers of this membrane are frequently heavily contaminated by various microörganisms, it became necessary to develop a technique of reaching the deep layers of the lingual mucous membrane. Following is a description of the techniques that have been developed. These were arrived at after many unsuccessful attempts by various means.

From the State Veterinary Research Institute, Amsterdam, Holland. The author is director of the institute.

The first paper of this series, "Histologic Changes in Explanted Bovine Epithelial Tongue Tissue Infected with the Virus of Foot-and-Mouth Disease," appeared in the American Journal of Veterinary Research, vol. x, No. 35, April, 1949, pp. 142-145.

METHODS

1) A tongue in a very fresh condition is desirable for collection of the epithelium (fig. 1). Although cooled tongues may be used, the results are less favorable than those from warm, fresh tongues. Moreover, the removal of the outer layers of epithelium is more difficult when cooled tongues are used. The tongues should preferably be taken from animals highly susceptible to foot-and-mouth disease. The use of tissue from animals that have been previously infected with the disease, even long before, may result in poor virus production or none at all.

2) The tongue is cleaned with a brush and warm water.

3) The tongue is tightly fixed at its tip in a clamp on a stand, so that it hangs at full length with the base downward (fig. 2). With some practice, after having incised the mucosa around the tip of the tongue, one may separate the superficial from the deep layers of the epithelium at the tip. Separation of these layers is best started with the blunt edge of the handle of a scalpel. After separation for a distance of about 3 cm., the incision is extended along the lateral margins of the dorsal surface of the organ.

4) The mucous membrane is washed again, this time with alcohol (70%). The loosened part of the membrane is fixed in a specially designed clamp, as shown in fig. 3, 4, and 5.

5) The separated exterior portion of the membrane is fixed in the clamp and rolled around this instrument, thus uncovering the underlying deeper layers of uncontaminated epithelial tissue.

6) The underlying mucosa consists of the stratum germinativum and some layers of the stratum spinosum. The mucosa is sliced from the exposed tongue surface with a sterile, sharp safety razor blade, mounted in a sterilized handle. The muscular tissue of the tongue must be avoided, since lactic acid, which is nocuous to the virus, is formed during the incubating period.

7) The fragments of epithelial tissue, with the attending small quantity of connective

Fig. 1—Tongue ready for collection of the epithelial tissue.

Fig. 2—The tip of the mucosa fixed in the clamp to be rolled down.

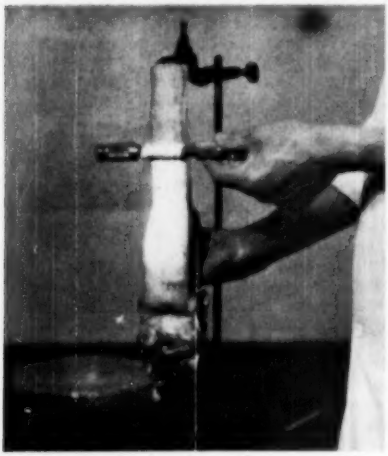


Fig. 3—Sterile, deep layer of mucosa visible.

tissue, are placed in Tyrode's solution and finely minced with special scissors.

8) To about 4 Gm. of the minced tissue is added approximately 15 ml. of virus diluted 1 : 2 with isotonic buffer solution (Na_2HPO_4 and NaH_2PO_4) pH 7.6. Five drops of a solution containing 100,000 units of penicillin in 20 ml. of water and 5 drops of a solution containing 1 Gm. of streptomycin in 20 ml. of water are included to insure bacteriostasis without damage to the cells. To these is added 125 ml. of Baker's fluid.[12] The whole quantity is placed in a wide-necked Erlenmeyer flask arranged for aëration with carbogen (95% O_2 and 5% CO_2).

9) After incubation for twenty-four hours at 37 C., the virus is harvested by grinding the tissue with sterile quartz sand in a mortar, resuspending it in the medium that was used for cultivation, and finally separating it from the tissue by centrifugation. The supernatant fluid is then filtered through asbestos disks.

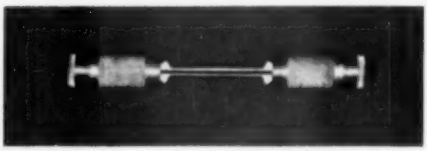


Fig. 4—Clamp used for removing superficial layer of mucosa.

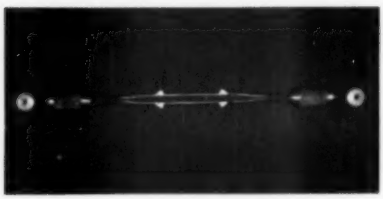


Fig. 5—Construction of the clamp.

DISCUSSION

Although there is no appreciable multiplication of cells in the epithelial tissue, sufficient cells survive to propagate the virus in large quantities. Generally, the concentration of the virus is as great as in tissue from inoculated animals. The freshness of the tongue and the composition of the nutrient medium play an important part in the production of the virus.

Titrations of the virus are conducted in cattle following the method described by Henderson.[13] Of each dilution, ten intradermic injections are made on the dorsal

surface of the tongue, the greater dilution being injected anteriorly. The titer (50% end point) of the culture virus is determined after the method of Reed and Muench.[14] The titer usually varies from 10^{-5} to 10^{-6} and sometimes slightly higher.

By the methods described, we have transferred an A type virus through more than 200 serial passages. More than 150 passages of an O type virus have been made.

Histologic examinations indicate that the lesions in the cells[11] may occur after twelve to sixteen hours of incubation. Long-continued incubation is detrimental to the virus, and the indications are that the shorter the period, the better the virus yield.

One tongue from an adult bovine animal yields an average of 4 to 8 Gm. of epithelial tissue. However, in addition, it has been found feasible to remove a portion of the tissue from the inner side of the upper layers of the epithelium that have been separated by means of the clamp. About 250 ml. of virus may be cultivated from one tongue.

Much detailed experimentation remains to be done in order to determine the optimum and most practical procedures. However, sufficient progress has been made to indicate that virus cultivated by this means may supplant the virus from living animals that is used in the present vaccine. With the reduced costs and the greater safety of laboratory procedures, resulting from the use of this method, the fight against this dread malady will be more effective.

SUMMARY

A method is described for the cultivation of the virus of foot-and-mouth disease in surviving explanted epithelial tissue taken from the tongues of adult susceptible cattle.

The titer of the virus usually ranges from 10^{-5} to 10^{-6}.

The average quantity of virus obtained from the 4 to 8 Gm. of tissue harvested from each adult bovine tongue is 250 ml.

References

[1]Frenkel, H. S., en van Waveren, G. M.: Over het kweken van monden-Klauwzeervirus in vitro. Uitgave Algemene Landsdrukkerij, 's-Gravenhage, 1935.

[2]Frenkel, H. S., en van Waveren, G. M.: Over het kweken van monden-klauwzeerstetstof op huidexplantaten van runder-en schapen-embryos. 2e mededeling. Uitgave Algemene Landsdrukkerij, 's-Gravenhage, 1935.

[3]Frenkel, H. S., et van Waveren, G. M.: La culture du virus de la fievre aphteuse dans les explanations de la peau embryonnaire porcine. Bull. Off. internat. Epizoot., *14*, (1937): 351-352.

[4]Frenkel, H. S., en van Waveren, G. M.: Jaarverslag van het Staats Veeartsenijkundig Onderzoekings Institut, 1936-1937.

[5]Frenkel, H. S., en van Waveren, G. M.: Jaarverslag van het Staats Veeartsenijkundig Onderzoekings Institut, 1936-1937.

[6]Frenkel, H. S.: Der jetzige Stand der Züchtung von Maul-und-Klauenseuchevirus. Schweiz. Ztschr. f. Allg. Path. und Bakt., *1*, (1938): 214-227.

[7]Frenkel, H. S., and van Waveren, G. M.: Sulla coltura del virus aftoso negli esplantati di cute di bue e di pecora. Biochimic. e terap. sper., *23*, (1936): 279-282.

[8]Maitland, Mary Cowen, and Maitland, H. B.: Fourth Progress Rep. British Foot-and-Mouth Dis. Res. Committee, (1931): 345-359.

[9]Hecke, Friedrich: Züchtungsversuche des Maul- und Klauenseuchevirus in Gewebskulturen. Zentralbl. f. Bakt., *116*, (1930): 386-414, *119*, (1931): 385-397.

[10]Hecke, Friedrich: Zentralbl. f. Bakt., *128*, (1933): 336.

[11]Frenkel, H. S.: Histologic Changes in Explanted Bovine Epithelial Tongue Tissue Infected with the Virus of Foot-and-Mouth Disease. Am. J. Vet. Res., *10*, (1949): 142-145.

[12]Parker, Raymond C.: Methods of Tissue Culture. Paul B. Hoeber, Inc., New York, (1938): 76.

[13]Henderson, W. M.: The Quantitative Study of Foot-and-Mouth Disease Virus. A.R.C. Rep. Series No. 8, Agric. Res. Council, 1949.

[14]Reed, L. J., and Muench, H.: Simple Method of Estimating 50 Per Cent Endpoints. Am. J. Hyg., *27*, (1938): 493-497.

Preliminary Studies on the Effect of Streptomycin and Other Agents on *Mycobacterium Paratuberculosis*

A. B. LARSEN, D.V.M., T. H. VARDAMAN, M.S., D.V.M., and A. H. GROTH, M.S., D.V.M.

Auburn, Alabama

PARATUBERCULOSIS (Johne's disease) is a chronic infectious disease of cattle, sheep, and goats. The chief symptom is a chronic intermittent diarrhea without straining. There is a gradual loss of flesh, even though the appetite may remain good. Infected animals showing clinical symptoms usually die after a variable period of time. A thickening and corrugating of the intestinal mucosa near the ileocecal valve are usually found on postmortem examination of the animals. The causative organism, *Mycobacterium paratuberculosis*, is a small, rod-shaped, acid-fast bacillus. It is often found in large numbers in the diseased mucosa and in adjacent mesenteric lymph glands. Its morphology is so similar to that of the tubercle bacillus that positive differentiation under the microscope is difficult or impossible.

During recent years, the treatment of tuberculosis in human beings with antibiotics and other agents has received considerable attention. Since *Mycobacterium tuberculosis* and *M. paratuberculosis* are similar in morphology and cultural habits, it was considered desirable to study the effectiveness of some of these products against *M. paratuberculosis*.

Streptomycin appears to be the most popular and effective agent now available for the treatment of human tuberculosis. Patients in the early stages of tuberculosis respond more readily to treatment with streptomycin than do patients who are in the advanced stages of the disease.[1] Clinical improvement, when it occurs, is prompt and obvious within the first two weeks of treatment.[2] In a study of 745 positive cultures of *M. tuberculosis* isolated from patients, 97 per cent failed to grow on mediums containing 1 μg. of streptomycin per cubic centimeter.[3]

The opinion has been advanced that, in the treatment of tuberculosis, lesions of the mucous membranes respond more readily to streptomycin treatment than do lesions of the parenchyma.[4] Paratuberculosis being primarily a disease of mucous membranes, it seemed probable that it might be favorably influenced by streptomycin. Johnson[5] found that 14 μg. of streptomycin per cubic centimeter of liquid medium prevented growth of a culture of *M. paratuberculosis*.

A cow showing clinical evidence of Johne's disease was treated with 150 Gm. of streptomycin over a five-day period by McManus.[6] The results were unsatisfactory because the cow gradually became worse and was destroyed several months later.

In addition to streptomycin, other agents were investigated for their inhibitory effect on the growth of cultures of *M. paratuberculosis*. These were promin, 4-aminosalicylic acid, stilbamidine diisethionate, and pentamidine isethionate.

Promin has been used with good results in the treatment of leprosy.[7, 8] The disease is caused by *Mycobacterium leprae*, which is also morphologically similar to *M. paratuberculosis*.

Since streptomycin was the only one of these agents which showed promise in *in vitro* studies, it was selected for *in vivo* studies to determine its possible effect on clinical cases of Johne's disease. Because of the expense and the small number of clinical cases available, only preliminary *in vivo* trials could be made at this time.

IN VITRO STUDIES

Procedure.—These studies were made on a liquid medium prepared from the following ingredients:

Asparagine	14.0 Gm.
Dipotassium phosphate	1.8 Gm.
Sodium citrate	0.9 Gm.
Magnesium sulfate	1.5 Gm.
Ferric citrate	0.3 Gm.
Dextrose	10.0 Gm.
Glycerin	100.0 cc.
Distilled water to make	1,000.0 cc.

The medium was placed in 300-cc. flasks in 100-cc. amounts, sterilized, and inoculated with strain 18 of *M. paratuberculosis*. Seven days later, flasks with uniform growth were selected for the titrations. Each dilution of each agent tested was added to three flasks.

From the Regional Animal Disease Research Laboratory, Bureau of Animal Industry, Agricultural Research Administration, United States Department of Agriculture, Auburn, Ala.

Additional flasks with an equal amount of growth were used as controls for each dilution. All flasks were handled alike and were kept side by side in the incubator at 37 C.

The dilutions of the materials tested ranged from 2,000 μg. per cubic centimeter of medium to 0.5 μg. per cubic centimeter of medium. All flasks were observed three times a week for one month after adding the agents being tested. If no further growth had occurred by that time in a particular flask, the growth in that flask was recorded as having been inhibited by the agent being tested in the flask.

RESULTS

The results of the *in vitro* experiments are recorded in table 1. Pentamidine isethionate prevented growth of *M. paratuberculosis* when the medium contained 2,000 μg. per cubic centimeter, but it did not prevent growth when the medium contained 500 μg.

Promin, 4-aminosalicylic acid, and stilbamidine all prevented growth of *M. paratuberculosis* when the medium contained 500 μg. or more per cubic centimeter. They did not prevent growth when the medium contained 125 μg. or less. No further growth of *M. paratuberculosis* cultures occurred in flasks containing more than 1 μg. of streptomycin per cubic centimeter of medium. However, the cultures continued to grow in two out of three flasks containing 1 μg. of streptomycin per cubic centimeter of medium and in all flasks containing 0.5 μg. The cultures in all control flasks grew at a normal rate.

IN VIVO STUDIES

Procedure.—Since streptomycin gave the most promising results in *in vitro* studies, plans were made to study its effect on clinical cases of paratuberculosis. Two heifers and 2 cows showing clinical symptoms of the disease were used in this study. The diagnosis was confirmed by a positive intradermal johnin test and by the presence of typical acid-fast organisms in rectal scrapings when examined under the microscope.

The course of treatment varied with each animal. The dosage ranged from 2 Gm. per 100 lb. of body weight in animal 2, to 6 Gm. per 100 lb. of body weight in animal 4. The streptomycin was dissolved in sterile saline, divided into several doses, and administered intramuscularly at six-hour intervals. The length of time the animals were under treatment varied from twenty-four hours for animal 2, to sixty hours for animal 3. Details of the dosage are shown in table 2.

In order to determine if a blood level of streptomycin sufficient to inhibit the growth of *M. paratuberculosis in vitro* was obtained, blood was drawn under sterile conditions from animal 1 during the course of treatment. This blood was defibrinated and added to three flasks of liquid medium containing two-week-old growing cultures of *M. paratuberculosis*. The blood was added at the rate of 1 cc. to each 4 cc. of medium. Blood was also drawn from an untreated animal, defibrinated, and added to three additional flasks in the same manner.

OBSERVATIONS AND RESULTS

The animals were closely observed during and after treatment. No toxic effects were noticed. All animals showed marked improvement within twenty-four hours following treatment. However, the improvement was temporary, because symptoms recurred in all cases from three to four weeks after treatment. Animals 1 and 2 died forty-eight and seventy-one days, respectively, after treatment. Animals 3 and 4 were destroyed seventy and thirty-six days, respectively,

TABLE 1—Comparison of the Inhibitory Effect of Various Agents on *Mycobacterium Paratuberculosis* Cultures

Agent tested	No. of cultures in each dilution	No. of cultures showing growth in each concentration of agent tested (μg. per cc.)								Total cultures
		2,000.0	500.0	125.0	31.0	7.5	3.75	2.0	1.0	0.5	
None (Control flasks)	6	6	6	6	6	6	6	6	6	6	54
Streptomycin	3	0	0	0	0	0	0	0	2	3	27
4-amino-sallcylic acid	3	0	0	3	3	3	3	3	3	3	27
Promin	3	0	0	3	3	3	3	3	3	3	27
Stilbamidine	3	0	0	3	3	3	3	3	3	3	27
Pentamidine isethionate	3	0	3	3	3	3	3	3	3	3	27

after treatment because they were considered beyond recovery.

In the cultures to which the defibrinated blood of animal 1 was added, no further growth was observed. However, in the flasks containing defibrinated blood from an untreated animal, the cultures continued to grow at the same rate as untreated cultures kept as controls.

DISCUSSION

Promin, 4-aminosalicylic acid, stilbamidine diisethionate, and pentamidine isethionate had to be added to the medium in relatively large amounts before any of them prevented growth of *M. paratuberculosis*. It was concluded that none of the foregoing materials showed enough promise to warrant using them on the clinical material available.

The results of the *in vitro* studies with streptomycin demonstrated that it prevented growth of a strain of *M. paratuberculosis* when added at the rate of 1 μg. per cubic centimeter to medium inoculated with *M. paratuberculosis*. A blood level of streptomycin high enough to prevent growth of *M. paratuberculosis* cultures was obtained in an animal under treatment; however, the results with streptomycin in the treatment of clinical cases of paratuberculosis were not encouraging, as observed on a limited number of cases. In all of the cases, the improvement was temporary and no recoveries resulted. The clinical cases used in these *in vivo* investigations were in advanced stages of the disease. If therapy could be initiated shortly after the animal becomes infected and continued for an extended period, the results might be more encouraging than those reported here. However, treating a large animal with the required quantity of streptomycin would be expensive and not practicable under ordinary conditions.

SUMMARY

1) *In vitro* studies of promin, 4-aminosalicylic acid, stilbamidine diisethionate, pentamidine isethionate, and streptomycin indicate that streptomycin was the only one of these agents that inhibited the growth of cultures of *Mycobacterium paratuberculosis* in low concentrations.

2) It was possible to produce, in a cow under treatment, a blood level of streptomycin high enough to inhibit growth of *M. paratuberculosis* cultures, when blood from the treated animal was added to the growing cultures. (This animal had received 2.5

TABLE 2—Symptoms, Dosage, and Effect of Streptomycin Treatment on Clinical Cases of Paratuberculosis

Animal (No.)	Breed	Body weight (lb.)	Age (yr.)	Results of microscopic examination	Symptoms	Date symptoms first observed	Date of treatment	Streptomycin dosage	Effect of treatment	Final result
1	Jersey	420	2	+	Marked scouring, loss of weight.	9- 1-48	9- 2-48	10 Gm. over a 24-hour period.	Marked temporary improvement, then recurrence of symptoms.	Death 48 days after treatment.
2	Jersey	410	2	+	Marked scouring, loss of weight.	10-22-48	10-22-48	8 Gm. over a 24-hour period.	Marked temporary improvement, then recurrence of symptoms.	Death 71 days after treatment.
3	Jersey	600	11	+	Marked scouring, rapid loss of weight.	2-15-49	3- 3-49	25 Gm. over a 60-hour period.	Marked improvement, then gradual recurrence of symptoms.	Destroyed as incurable 70 days after treatment.
4	Shorthorn	575	4	+	Marked scouring, rapid loss of weight.	4-15-49	6- 6-49	36 Gm. over a 48-hour period.	Slight improvement, then gradual recurrence of symptoms.	Destroyed as incurable 36 days after treatment.

Gm. of streptomycin per 100 lb. of body weight during the 24-hour period.)

3) Streptomycin, when administered in doses not exceeding 36 Gm. and over periods not exceeding sixty hours, produced temporary improvement in cows showing clinical symptoms of paratuberculosis. Improvement ceased three to four weeks after treatment was discontinued, and the animals subsequently died or were destroyed as incurable. However, more complete investigations are needed before final conclusions are reached.* Such studies should include animals in various stages of the disease and reactors not showing clinical symptoms.

*While this paper was being prepared, Dr. D. C. Bostwick (personal communication) treated 2 cows, both reactors to intradermal johnin, each receiving 25 Gm. of streptomycin daily for seven days. At the time of treatment, neither of these animals was showing clinical evidence of Johne's disease, although acid-fast bacilli were demonstrated in rectal scrapings. Four months after treatment, these animals were still reactors, and acid-fast organisms were present in rectal scrapings.

References

[1]Streptomycin in the Treatment of Tuberculosis. Report to the Council on Pharmacy and Chemistry. J. A. M. A., *138*, (1948):584.

[2]Johnson, M. P.: Paratuberculosis or Pseudotuberculosis Enteritis of Cattle. Virginia Agric. Exper. Sta., Bull. 419, (1947):15 pp.

[3]McManus, N. R., Catskill, N. Y.: Personal communication.

[4]Cowdry, E. V., and Ruangsiri, C.: Influence of Promin, Starch and Haptaldehyde on Experimental Leprosy in Rats. Arch. Path., *32*, (1941):632.

[5]Faget, G. H., Pogge, R. C., Johansen, F. A., Dinan, J. F., Pregean, B. M., and Eccles, C. G.: The Promin Treatment of Leprosy. Pub. Health Rep., *58*, (1943):1729.

Toxicologic Studies of Aldrin (Compound 118) on Large Animals

C. H. KITSELMAN, V.M.D., M.S., P. A. DAHM, Ph.D., and A. R. BORGMANN, D.V.M., M.S.

Manhattan, Kansas

ALDRIN* is the coined name for a chlorinated hydrocarbon developed as an insecticide by Julius Hyman and Co. This product has proved to be highly efficacious in the control of certain insects such as grasshoppers and boll weevils.

The purpose of the experiments was to study the acute, subacute, and chronic toxicity of aldrin to a number of the larger experimental animals. Steers, heifers, dairy cows, and sheep were selected for the studies.

Aldrin was administered to the animals in the form of a residue remaining on alfalfa hay that had been treated in the field at the rate of 0.5 lb. of aldrin per acre, by the addition to the daily ration of known amounts of aldrin dissolved in corn oil, and by oral administration to the animal of a solution of aldrin in corn oil.

Various tissues, urine, and feces, as well as the milk from the dairy cows, were analyzed for aldrin. Biologic assays[1] were carried out with adult houseflies, and the chemical analyses were made by the use of the Danish-Lidov[2] colorimetric procedure. Histopathologic studies of the tissues from the experimental animals were also made.

The alfalfa used in these studies was treated with aldrin in the field at the rate of 0.5 lb. per acre. The alfalfa was cut eight days after the application of the insecticide, dried and baled in a normal manner, and stored in a barn from which it was fed to the appropriate animals. The treated and untreated hay was substituted for normal hay as the sole source of roughage for certain animals. Alfalfa hay, comparable in quality to the sprayed hay cut from part of the same field where the hay was not treated, was fed to certain animals for weight gains and other comparisons. Several samples were taken from the treated alfalfa during the course of the experiment and the residues of aldrin were estimated by means of biologic assays with houseflies; by this procedure, not more than 8 parts per million (p.p.m.) of aldrin were found on the treated alfalfa at any time.

From the Departments of Veterinary Medicine and Entomology, Agricultural Experiment Station, Kansas State College, Manhattan.

Contributions No. 114, Department of Veterinary Medicine, and No. 575, Department of Entomology.

This report covers the large animal phase of a cooperative investigation involving the Departments of Veterinary Medicine and Entomology. A report on the small animal phase of the investigation will appear at an early date. The work was supported by a research grant established at the Kansas Agricultural Experiment Station, Sept. 1, 1948, by Julius Hyman and Co., Denver, Colo. The coördinating committee consists of R. I. Throckmorton, E. E. Leasure, Roger C. Smith, C. H. Kitselman, A. R. Borgmann, and Paul A. Dahm.

The authors wish to thank Dr. E. R. Frank and Dr. E. J. Splitter of the School of Veterinary Medicine, and graduate assistants J. E. Pankaskie and Yao Hsiung of the Department of Entomology for assistance in carrying out various phases of the work.

*Aldrin as used in these experiments applies to the recrystallized product containing not less than 99 per cent of the compound.

EXPERIMENT 1

Eight nonpregnant and 2 pregnant ewes were used to study the effect of aldrin on sheep. After sufficient time had elapsed for them to become acclimated, they were separated into groups. One group, consisting of 4 ewes, was fed daily for 169 consecutive days a ration containing approximately 4 lb. of aldrin-treated hay per animal. At the end of this period, 1 ewe was sacrificed for autopsy and to furnish tissues for bio-assay and chemical examination. Two of the ewes were slaughtered ninety days after their removal from the ration containing aldrin, and the fourth ewe was slaughtered 120 days after her return to an aldrin-free ration. Tissues, particularly the internal organs and specimens of body fat, were removed for bio-assay and chemical examination to determine quantitatively the presence of aldrin in the organ or carcass. This was essentially a search for residual aldrin after time had elapsed for the body to free itself of the compound.

The sheep designated as controls ate a ration identical to that of the sprayed-hay group, except that it contained no aldrin. These animals were used for purposes of comparison, particularly regarding gain or loss in body weight and quantities of hay consumed.

To obtain a more exact dosage of aldrin, 1 ewe was given single, daily dosages of the compound dissolved in corn oil and mixed with the dry portion of the ration, at the rate of 6 mg. of aldrin per kilogram of body weight. After receiving 15 doses of the treated feed, the animal refused to eat and the subsequent doses were given as a drench. On the twenty-eighth day following the start of this phase of the experiment, the ewe showed symptoms of

TABLE 1—The Effect on Health and Tissue Analysis of Animals Fed Aldrin-Sprayed Hay

	Animal	No. days fed sprayed hay	Effect on health	Disposition of animal	Tissue	Biologic assay* p.p.m. of aldrin average (2 assays)
Ewe	4032	169	None	Slaughter	Fat	0.58
	4034	169	None	Slaughter	Fat	2.00
					Liver	3.90
					Kidney	1.50
					Brain	1.60
	4035	169	None	Slaughter	Omentum fat	1.00
					Subcutaneous fat	1.00
	4036	169	None	Slaughter	Omentum fat	0.00
Heifer	4021	169	None	Slaughter	Fat	T**
					Liver	0.60
					Kidney	0.50
					Brain	0.80
	4024	213	None	Fat obtained by biopsy	Omentum fat	0.20
	4026	213	None	Fat obtained by biopsy	Omentum fat	0.50
	4021	213	None	Fat obtained by biopsy	Omentum fat	T**
Cow	93456	91	None	No fat sample obtainable***		

*An accurate chemical test for aldrin was not available when this work was done.
**Trace. ***Cow returned from pasture in an emaciated condition.

TABLE 2—The Effect on Health and Tissue Analysis of Animals Given Measured Doses of Aldrin

Animal	Daily dose (mg./kg.)	Total aldrin consumed (Gm.)	No. days fed	Effect on health	Biologic assay* p.p.m. aldrin average (2 assays)						
					Fat	Pancreas	Kidney	Liver	Spleen	Brain	Bile
Steer 4022	1.960	19.00	10	Death	38.8	10	11	11	12.5	18	7.6
Ewe 4039	0.414	8.00	20	Death	28.8	11	8	18	12.5	20	7.5
Heifer 4042	0.840	17.68	21	Death	1.4 (blood)		10	27	0.9	12	2.7 (muscle)
Cow 752539	0.686	22.00	33	Death	22.0		15	19		16	
Heifer 4023	0.520	33.28	64	Symptoms on 29th day.** Recovery on 50th day. Fat obtained by biopsy.	0.9						
Heifer 4027	0.290	18.56	64	None. fat sample by biopsy.	0.9						
Heifer 4028	0.113	7.23	64	None. fat sample by biopsy.	T***						

*An accurate chemical test for aldrin was not available when this work was done.
**Disturbance of the central nervous system. ***Trace.

incoördination, leg weakness, and hypersensitivity to external stimuli, such as sudden noise or the touch of a hand or a stick. The animal died two days after the symptoms appeared. Samples of the vital organs and the body fat were removed for bio-assay and chemical examination.

A dog fed the organs and meat from this ewe died from aldrin poisoning. Biologic assay of the liver and the brain from this dog showed 12 p.p.m. of aldrin in each organ.

EXPERIMENT 2

Twelve head of cattle of mixed beef breeds, ranging from 350 to 500 lb. in weight, were obtained for the study of the effect of aldrin on growth as well as the acute and subacute dosage range. Two lactating Jersey cows were used to study the effect of aldrin on milk secretion, particularly to find the rate of elimination compared to the storage rate on a measured intake of the chemical. Four heifers were placed on a ration containing approximately 9 lb. of the aldrin-treated alfalfa hay per animal, per day. All of these animals ate this ration for 213 days. At the end of this period, 1 heifer was slaughtered and the vital organs with specimens of the body fat were removed for laboratory examination. The 3 remaining heifers were placed on fresh pasture, free from insecticidal residues, for ninety-six days. Following the pasture season, samples of fat, approximately 100 Gm. in weight, were obtained by biopsy from each of these heifers. These samples were taken from the omentum and were examined both by biologic assay and chemical analysis for residues of aldrin.

The cattle designated as controls ate a ration identical with that of the sprayed-hay group, except that it contained no aldrin-treated hay. These animals were used for comparison, particularly in their rate of growth and hay consumption.

In order to obtain a more exact dosage of aldrin, 5 of the beef cattle were fed known amounts of aldrin dissolved in corn oil and incorporated in the dry portion of the ration fed to these animals. A steer was fed aldrin daily at the rate of 7.5 mg. per kilogram of body weight. On the eighth day, the steer showed hyperirritability followed by incoördination and leg weakness. Convulsions followed and the animal died on the tenth day. Approximately, 19 Gm. of aldrin had been consumed.

EXPERIMENT 3

A heifer, fed aldrin at the rate of 4 mg. per kilogram of body weight as a daily dose, developed symptoms of poisoning on the seventh day, similar to those of the steer. This animal developed convulsions and died on the twenty-first day. A total of 17.7 Gm. of aldrin was consumed.

Another heifer was fed daily doses of aldrin at the rate of 2 mg. per kilogram of body weight. Symptoms similar to those observed in the steer and heifer also were observed in this animal but did not appear until the twenty-ninth day. In spite of the fact that this heifer continued to ingest her allotted dose of aldrin, she had returned completely to good health by the fiftieth day and, after sixty-four days of continued feeding of the compound, was turned out on clean pasture for the grazing season. A total of 33.28 Gm. of aldrin was consumed by this animal. After she remained on pasture for one hundred days, a sample of fat was obtained by biopsy for the laboratory examination for residual aldrin.

A third heifer was fed aldrin at the rate of 1 mg. per kilogram of body weight per day for a period of sixty-four days. At no time during the feeding, or later, were symptoms of aldrin poisoning observed in this animal. After one hundred days in a clean pasture, a sample of omental fat was obtained by biopsy from this animal and examined, both by the chemical and biologic assay methods. A total of 18.56 Gm. of aldrin was consumed during the feeding period.

The fourth heifer was fed aldrin at a dosage level of 0.5 mg. per kilogram of body weight for a 64-day period. This animal did not show any evidence of aldrin poisoning at any time. Following a pasture season of one hundred days, a fat sample was obtained by biopsy for laboratory examination. A total of 7.23 Gm. of aldrin was consumed during the feeding period.

One of the Jersey cows was fed aldrin-treated hay for a period of five months without showing any evidence of poisoning. This cow had been bred for one month before the start of the feeding experiment and produced a normal calf three months after returning to a ration free from the compound. A second Jersey cow was fed aldrin at the dosage level of 2 mg. per kilogram of body weight, per day. Symptoms identical to those formerly described for this type of poisoning in cattle appeared on the eighteenth day. Milk secretion rapidly decreased as emaciation of the animal increased. Death occurred on the forty-first day. The total amount of aldrin consumed was 22 Gm.

A dog fed the organs and part of the meat (skeletal muscles) of this cow died from aldrin poisoning. Biologic assay of the liver and the brain of the dog showed 8.2 and 10.0 p.p.m. of aldrin, respectively.

RESULTS

The results of these studies can be summarized as follows.

1) Neither the sheep nor the cattle fed aldrin-sprayed alfalfa hay showed any toxic

effect during the feeding periods of 169 and 213 days, respectively.

2) Less than 4 parts per million (p.p.m.) of aldrin were found in the liver and only 2 p.p.m. of aldrin were found in the body fat of a ewe eating aldrin-treated hay for a period of 169 days. There were no symptoms of poisoning at any time in this animal. Fat samples, obtained by biopsy from the beef cattle fed aldrin-treated hay for 213 days and then pastured on aldrin-free grass for three months, proved to contain less than 1 p.p.m. of aldrin.

3) There was less than 1 p.p.m. of aldrin found in the tissue examined (liver, kidney, brain, and body fat) of the heifer fed aldrin-treated hay for a period of 169 days.

4) In the tissues of the animals which died after eating aldrin fed at daily levels ranging downward from 7.5 to 2.0 mg. per kilogram of body weight, the chemical was recovered in amounts ranging from 50 p.p.m. to less than 1 p.p.m. The body and the perirenal fat contained the largest amount of the compound. This group consisted of 1 steer, 1 heifer, 1 Jersey cow, and 1 ewe. The omental fat of 3 heifers fed aldrin at levels of 2.0, 1.0, and 0.5 mg. per kilogram of body weight, respectively, during a period of sixty-four days contained less than 1 p.p.m. when biopsied after the heifers grazed one hundred days on a pasture free from aldrin.

5) The cattle in the sprayed-hay group showed the same average gain in body weight as those eating hay comparable in quality but free from aldrin.

6) The milk from the Jersey cow fed aldrin-treated alfalfa hay did not show any trace of aldrin at any time during the feeding period (91 days).

7) Degenerative changes in the kidney and liver were uniformly observed in those animals dying from measured dosages of aldrin.

Various details are summarized in tables 1 and 2.

References

[1]Dahm, P. A., and Pankaskie, J. E.: A Biological Assay Method for Determining Aldrin. J. Econ. Entomol., 42, (1949) 987-988.

[2]Danish, A. A., and Lidov, Rex.: A Colorimetric Procedure for the Determination of Compound 118. Presented at the 115th Nat. Meet. of the Am. Chem. Soc., San Francisco, March 30, 1949. Unpublished.

Studies on Johnin

VIII. Preliminary Studies on the Specificities of Acid-Fast Allergens

L. A. BAISDEN, M.S., Ph.D., H. W. JOHNSON, M.S., D.V.M., and A. B. LARSEN, D.V.M.

Washington, D. C., and Auburn, Alabama

BUREAU OF ANIMAL INDUSTRY (BAI) intradermal johnins and tuberculins have been shown to contain nonspecific factors which cause them to elicit reactions when injected into cows sensitized to other than homologous acid-fast microorganisms.[1] Attempts to increase the specificity of these allergens have been made in the Bureau laboratories for a number of years. As a basis for evaluating the changes in specificities, methods for assaying potencies of tuberculins and johnins have been developed and described by Johnson[2] and Wadley[3,4] working in the Department of Agriculture. This paper presents briefly the results obtained in a number of attempts to modify the specificities of johnins and tuberculins.

The general plan of the work was to subject BAI intradermal johnins and BAI human and avian tuberculins to various treatments known to affect proteins and then determine which treatments had affected the specificities of the treated materials in comparison with those of the original allergens. The comparisons were made by simultaneous injections of the derived and original materials into cows artificially sensitized to heat-killed *Mycobacterium paratuberculosis* or *Mycobacterium tuberculosis*.

MATERIALS AND METHODS

Treatments. — The treatments used were chemical, physical, and biologic. Further details of the treatments are given in the results obtained with the different products of the treatments.

Experimental Designs.—Two procedures, or designs, for testing were used in this work. They were the design used in the "potency unit" method of assaying the potencies of johnins as described by Johnson[2] and a statistical design described by Wadley[4] (p. 107).

The first testing procedure did not permit a very good evaluation of the variations due to the animal differences, concentration and material differences, and skin region differences. It, therefore, did not allow the estimation of experimental errors which could be used in determining the differences in specificity required for significance in the various experiments.

The second design allowed a good evaluation of the various known sources of variation and the estimation of the differences in specificities required for significance. Consequently, it was used in the later tests.

Both designs utilized the increase in skin thickness, as measured in millimeters, resulting from an injection as the fundamental measure of response. The skin thickness at the site of an injection was measured before the injection of a solution of the allergen under study and again forty-eight hours after the injection. Skin thickness measurements were made with the dermal thickness gauge described by Kummer and Johnson.[5]

Those experimental results which were evaluated by the potency unit method were observed on 3 to 5 homologously sensitized cows and the same number of heterologously sensitized cows. Each of 96 injections, 32 materials at three concentrations each, was located in the corresponding sites of each cow. Those experimental results which were evaluated by the statistical methods were taken on from 8 to 10 cows of each sensitization. Sixty-four injections, 32 materials at two concentrations each, or 16 materials at four concentrations each, were located on the skin of the animals in such a manner that all concentrations of the same material were kept adjacent to each other on all animals, but the order of placement of the materials themselves was "randomized" from cow to cow.

From the Pathological Division, Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture, Washington, D. C. (Baisden); and the Regional Animal Disease Research Laboratory, Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture, Auburn, Ala. (Johnson and Larsen).

The authors wish to acknowledge their indebtedness to Dr. F. M. Wadley, formerly of the Committee on Statistical Design of the U. S. Department of Agriculture, for designing the experiments carried out in the latter part of this work, for his encouragement and interest, and for his aid in studying the results. They also wish to express their appreciation for the interest and aid given by Mr. R. R. Henley of the Pathological Division, Bureau of Animal Industry, U. S. Department of Agriculture, Washington, D. C.

The parent, or original, BAI allergens used as standards in this work, as well as certain other materials, were tested on an Old Johnin (OJ)-Old Tuberculin (OT) basis.* Bureau of Animal Industry intradermal acid-fast allergens are used in field work at a strength of ¼ OT or OJ. Certain of the materials used in this work, especially solutions of precipitates, were tested on the basis of their nitrogen content. For purposes of comparison, a solution containing 0.1 per cent nitrogen was considered equivalent to a solution of ¼ OT or OJ strength.

Evaluating Specificities.—In both designs, the relative potencies of a material on homologously sensitized cows and on heterologously sensitized cows was the basis of evaluating its specificity.

In the evaluation of the potency of a material by the potency unit method, the total potency units were calculated by adding the products obtained by multiplying the dilution factor by the response in millimeters increase in skin thickness, for all concentrations on all cows. Specificity changes were detected by comparing the total potency units of an unknown with those of a standard on homologously sensitized cows and on heterologously sensitized cows. If the total potency units of an unknown equalled the total for the standard on the homologously sensitized cows and the total for the unknown were less than the total for the standard on heterologously sensitized cows, the unknown was deemed more specific than the standard.

For complete details of the statistical methods used, the reader is referred to the papers by Wadley already mentioned and to papers by Bliss and Marks[6] and Yates.[7]

The evaluation of potency by the statistical methods was based on the fact that a practically linear relationship existed between the logarithms of concentrations and the responses, which relationship permitted, with a fair degree of accuracy, the estimation of the concentration required to yield a certain response. Potency comparisons were made from the estimated logarithms of the concentrations of two materials which were required to produce equal responses. In a similar manner, the specificity of a material was estimated by comparing the logarithm of the concentrations required to give a certain response on homologously sensitized cows with that required to give the same response on heterologously sensitized cows. The differences between these two logarithms was the specificity factor, or the logarithm of the ratio of the potencies on the two sensitizations. A material which had a higher specificity factor or ratio of potencies on the two sensitizations than did the standard had a higher specificity.

Significance of differences in specificities was calculated by using the following formula:

$$s_m = \frac{s}{b\,av.}\sqrt{\frac{2}{n'}}$$

in which s is an experimental error which depended upon the cow variances for cows of both sensitizations and the error variances of both sensitizations, b av. is the slope of the lines relating logarithm of concentration to response for the two materials being compared, and n' is the total number of injections of any one material per sensitization.

TABLE I.—Effects of Various Treatments on the Specificities of Acid-Fast Allergens

Treatment	Effect on specificity of allergens		
	Johnin	Human tuberculin	Avian tuberculin
Protein precipitants	I	O	O
Ethyl alcohol fractionation:			
At high concentration	D	I	...
At low concentration	I	D	...
Surface active agents or detergents:			
Anionic	I	O	O
Cationic	O	I	O
Reducing agents	O	I	...
Oxidizing agent (chloramine-T)	O	O	...
Protein group reagents	O or D	O or D	O or D
Fractionation by ultrafiltration (acetic acid-collodion)	O	O	O
Ultraviolet irradiation	O	O	...
Variation of glycerol: nitrogen ratio of medium	...	O	...
Contamination with a mold and a bacterium	...	O	...
Pepsin	O	DD	...
Trypsin	D	DD	...

I = increase in specificity. For details concerning those treatments which increased specificity see text. O = no effect of importance. D = slight decrease. DD = marked decrease.

*Koch's Old Tuberculin strength is obtained by evaporating culture filtrate to 1/10 the volume of the original medium.

RESULTS

The results of these experiments are summarized briefly in table 1 and are given in detail below.

1) Treatments Which Produced Important Effects on the Specificity of BAI Intradermal Johnins and Tuberculins

A) Protein precipitants on johnin.

1) Trichloroacetic acid was used at a concentration of 8 per cent to produce precipitates of johnin protein. The precipitates were washed with 1 per cent solution of the same reagent, redissolved with the aid of a small amount of weak alkali, and tested on a nitrogen basis in six experiments, four by the potency unit design and two by the statistical design.
Results: In one of the statistically designed experiments, a considerable, though not significant, increase in specificity was observed, while in the others only a slight increase was observed.

2) Silicotungstic acid at a concentration of 0.8 per cent plus 2 per cent concentrated H_2SO_4 was used similarly in eight experiments.
Results: In one of two experiments of statistical design, a considerable increase in specificity was observed. In only four of the six experiments tested by the potency unit design were slight increases in specificity observed.

3) Acetone was used in a ratio of nine volumes to one to produce a precipitate which was tested by the statistical design in one experiment.
Result: A considerable increase, not significant, in specificity was produced.

4*a*) Ethyl alcohol at a concentration of 25 per cent and at varying acidities was used as a precipitant in one experiment tested by the potency unit design.
Result: A slight increase in specificity was shown by the fraction obtained at the high acidity of about pH 1.

4*b*) Ethyl alcohol was also used at a pH of about 6.0 and at 67.5, 75, 87.5, and 95 per cent concentrations in an experiment tested by the statistical design.
Result: A slight increase in specificity was shown by the precipitate at 67.5 per cent alcohol. Decreases in specificity were shown by the higher alcohol concentrations.

B) Ethyl alcohol fractionation of human tuberculin (BAI intradermal tuberculins used in the Bureau for testing cattle).
Human tuberculin was fractionated by precipitating with ethyl alcohol at 50, 67.5, 75, 87.5, and 95 per cent concentrations.
Results: In one experiment of statistical design, the fractions precipitated by the lower concentrations showed slightly decreased specificity, while the fraction precipitated by 95 per cent alcohol, and the final filtrate itself, showed slightly increased specificity as compared with the control.

C) Reducing agents on human tuberculin.

1) Sodium thiosulfate was added to a solution of a silicotungstic acid precipitate to make a final concentration of about 0.05 normal.
Results: The treatment caused a significant increase in specificity in one of three experiments of statistical design and caused increases in the other two experiments.

2) Sodium hydrosulfite was added to a solution of silicotungstic acid precipitate and the mixture held at room temperature for about two weeks. A precipitate developed, making the treatment objectionable.
Results: A slight increase in specificity was shown in the one experiment which was tested by the statistical design.

3) Sodium bisulfite solution was used to treat a silicotungstic acid precipitate in solid form for twelve hours. The precipitate was then washed and dissolved for test by the statistical design. The bisulfite was prepared by passing sulfur dioxide into a 0.5 normal sodium hydroxide solution until a pH of 2 was reached.
Result: A significant increase in specificity was produced.

D) A commercial anionic detergent on johnin and a commercial cationic detergent on human tuberculin.

1) The anionic detergent was used at a final concentration of 0.57 per cent to treat BAI johnin for a period of twelve to twenty-four hours. The resulting precipitate was discarded and the supernatant solution tested, without further changes, in three experiments. In another experiment, the potent material of the supernatant solution was precipitated by silicotungstic acid and redissolved in a smaller volume for test.
Results: Considerable, though not significant, increase in specificity was observed in two of three experiments

tested by the statistical design, while a slight increase was observed in the third. No change in specificity was observed in one experiment tested by the potency unit design. The potency was lowered in all experiments with the anionic detergent.

2) The cationic detergent was used at a final concentration of 0.14 per cent to treat BAI tuberculin by the same procedure used to treat johnin with the anionic detergent.

Results: In two of three experiments tested by the statistical design, significant increases in specificity were observed, while a slight decrease was observed in the third. No change was observed in the one experiment tested by the potency unit design. As was the case with the anionic detergent on johnin, the potency was lowered in all experiments with the cationic detergent.

E) Action of the enzymes pepsin and trypsin on johnin and human tuberculin proteins.

1) Quantities of johnin protein and human tuberculin protein were prepared by precipitation with nine volumes of acetone. Eighty-four milligrams of each protein were dissolved in 20 cc. of water and the pH of the two mixtures adjusted to 1.9 with HCl. Forty milligrams of crystalline pepsin and a small volume of toluene were added to each mixture. After incubation for twenty-four hours with occasional shaking, half of each mixture was removed, its pH readjusted to 7.1, and it was heated at 100 C. for fifteen minutes. The other half of each mixture was incubated five days before its pH was readjusted and it was heated.

Results: Both variations with pepsin decreased the specificity of tuberculin significantly in one experiment of statistical design. However, even the five-day treatment did not affect the specificity of johnin. The potencies on homologous sensitizations were not appreciably lowered.

2) Similar solutions of johnin and tuberculin proteins were treated with trypsin in the same manner except that the pH before incubation was adjusted to 7.5 with $NaHCO_3$.

Results: Highly significant decreases in the specificity of tuberculin were observed. A significant decrease was observed with johnin in the case of the five-day treatment, and a nearly significant decrease in the case of the one-day treatment. Marked lowering of the homologous potency of both proteins occurred.

2) Treatments Which Produced Unimportant Effects on the Specificities of BAI Acid-Fast Allergens

A) On human tuberculin.

Precipitation of protein with: acetic acid, trichloroacetic acid, silicotungstic acid, an anionic detergent, and lower concentrations of ethyl alcohol.

Refluxing with concentrations of formalin varying from 0.06 to 4 per cent.

Treatment with various protein group reagents[8,9]: erythrosin dye, nitrous acid, ninhydrin, mustard gas and its sulfone, and the mild oxidizing agent, chloramine-T.

Treatment with the protein denaturants: urea and guanidine.

The physical treatments: exposure to ultraviolet light and filtration through graded acetic acid-collodion filters.

The biologic treatments: contamination with a mold and a bacterium, modification of the glycerol-nitrogen ratio of the culture medium, and the modification of the physiologic state of the organisms by harvesting at various ages less than eleven weeks.

B) On avian tuberculin.

Precipitation by protein precipitants: acetic acid, trichloroacetic acid, silicotungstic acid, and the anionic and cationic detergents.

Refluxing at pH values 2.1, 3.5, and 8.5.

Refluxing with various concentrations of formalin.

Treatment with the protein group reagents: nitrous acid and ninhydrin.

Treatment with urea.

Treatment by the physical method of ultrafiltration.

C) On johnin.

Precipitation with protein precipitants: acetic acid and the cationic detergent.

Refluxing at pH values of 2.1, 3.5, and 8.5.

Refluxing with various concentrations of formalin.

Treatment with the protein group reagents: erythrosin dye, nitrous acid, ninhydrin, mustard gas and its sulfone, chloramine-T, and reducing agents such as sodium thiosulfate, sodium hydrosulfite, and sodium bisulfite.

Treatment with protein denaturants: urea and guanidine.

The physical treatments: exposure to ultraviolet light and filtration through graded ultrafilters.

The test of filtrate from young cultures.

DISCUSSION

Green and coworkers in England[9] and McIntosh and Konst in Canada[10] have re-

ported evidences of alterations in the specificities of acid-fast allergens, as determined by skin tests on guinea pigs. The chief importance of the findings of the experiments reported in this paper is that they indicate that the specificities of acid-fast allergens can be altered even when the resulting products are tested on artificially sensitized cows.

In none of the experiments in which the effects of precipitation on johnin were studied was a statistically significant increase in the specificity noted, but some increase was noted in 12 of the 16 experiments. The treatment appeared to effect a real increase in specificity. It is possible that the methods of assay were not precise enough to show significant increases for these materials in the individual experiments. Under the conditions of these tests —only one replication of a material per animal was used and inadequate attention was paid to the adjustment of concentrations—high slopes in the logarithm of concentration-response relationship were not obtained. Workers in England have obtained results which indicate that trichloroacetic acid precipitation of johnin increases its specificity.[11]

No good explanation of the fact that precipitation appears to increase specificity can be given at this time. It seems rather unexpected that any change in specificity should be effected, since it does not seem possible that much in the way of fractionation is accomplished, especially by such powerful precipitants as trichloroacetic acid, silicotungstic acid, and acetone. However, two explanations are suggested. The first is that at least a partial fractionation occurs, and that the larger molecules, which may be more susceptible to changes in pH and electric charges, are the more specific. The second is that during precipitation, with its resulting increased juxtaposition of the formerly dispersed molecules, some type of polymerization of the molecules takes place which covers up the nonspecific factors. In this connection, Seibert and coworkers[12] found that precipitation and concentration by ultrafiltration apparently cause polymerization of tuberculin protein molecules. Perhaps the first explanation offered is the more tenable, but attention is called to the indications noted in this work that the commercial anionic detergent produces a precipitate which when removed leaves a supernatant which is more specific than the original johnin.

It is noted in the section on "Results" that the johnin fraction obtainable by precipitation with 67.5 per cent ethyl alcohol showed increased specificity, while those obtained at higher concentrations showed reduced specificity. Just the reverse occurred with tuberculin, the increased specificity being found in the fractions obtained above 87.5 per cent and the reduced specificity in the fractions below this concentration. Thus, there is a suggestion that the larger molecules of johnin are the more specific, and that the smaller molecules of tuberculin are the more specific. Such a conclusion seems unwarranted in view of the fact that both pepsin and trypsin, which break larger protein molecules into component parts under conditions used in this work, were found to produce significant decreases in the specificity of tuberculin. The dependence of the specificity of johnin or tuberculin upon molecular size is thus highly uncertain and can be tested only by preparative methods giving accurate information as to molecular size. As indicated in the section on "Results," the use of Seibert's acetic acid-collodion ultrafilters[13] did not give consistent results.

The findings that reducing agents appear to increase the specificity of tuberculin and that proteolytic enzymes decrease its specificity encourage future attempts to determine the chemical basis of specificity or nonspecificity in acid-fast allergens. Although the latter treatment produced effects on both johnin and tuberculin opposite to those desired, the results indicate that specificity can be altered.

The differences in the activity on johnin and tuberculin proteins of the two enzymes further emphasized the differences in the two allergens.

The majority of the other treatments mentioned in the section on "Results" was tested in a number of variations and in a number of experiments, but none gave marked or consistent changes in specificity of the allergens.

SUMMARY

The main points of interest to be derived from these intradermal tests of treated

acid-fast allergens on artificially sensitized cows are as follows:

1) The specificity of Bureau of Animal Industry (BAI) intradermal johnins was apparently increased, though not significantly, in 12 of 16 experiments by precipitation with protein precipitants.

The increases appeared to be real but were not statistically significant because of the techniques used in testing. The consistency of the results indicates that the treatment furnishes a lead for further work.

2) Along with other evidence, the results obtained in fractionating BAI intradermal johnin and tuberculin with ethyl alcohol emphasized differences in the allergens. A fraction of johnin possessing increased specificity was precipitated by 67.5 per cent alcohol, while the fractions precipitated by higher concentrations possessed reduced specificities. The reverse was true with tuberculin, the concentrations greater than 87.5 per cent alcohol producing the precipitates of increased specificity.

3) The reducing agents, sodium thiosulfate, sodium hydrosulfite, and sodium bisulfite increased the specificity of a silicotungstic acid precipitate of BAI human tuberculin—in two cases significantly.

4) Pepsin produced significant decreases in the specificity of an acetone precipitate of tuberculin, but did not affect that of a similar precipitate of johnin. Trypsin decreased the specificities of precipitates of both tuberculin and johnin significantly.

5) The findings indicate that, as determined by tests on cattle, alterations of the specificities of acid-fast allergens are possible.

References

[1]Johnson, H. W., Larsen, A. B., Henley, R. R., and Groth, A. H.: Studies on Johnin. VI. The Relationship of the Allergens of *Mycobacterium Paratuberculosis*, *Mycobacterium Tuberculosis* var. *Avium*, *Bovis*, and *Hominis*, and *Mycobacterium Phlei*. Am. J. Vet. Res., *10*, (1949):138-141.

[2]Johnson, H. W.: Studies on Johnin. III. Multiple Testing on Sensitized Cattle as a Method of Studying Johnin Potency. Am. J. Vet. Res., *5*, (1944):179-188.

[3]Wadley, F. M.: Experimental Design in Comparison of Allergens on Cattle. Biometrics, *4*, (1948):100-108.

[4]Wadley, F. M.: The Use of Biometric Methods in Comparison of Acid-Fast Allergens. Am. Rev. Tuberc., *60*, (1949):131-139.

[5]Kummer, F. A., and Johnson, H. W.: Studies on Johnin. IV. Dermal Thickness Gauge. Am. J. Vet. Res., *5*, (1944):189-192.

[6]Bliss, C. I., and Marks, H. P.: Biological Assay of Insulin. Quart. J. Pharm. & Pharmacol., *12*, (1939):82-110, 182-205.

[7]Yates, F.: The Recovery of Interblock Information in Balanced Incomplete Block Designs. Ann. Eugenics, *10*, (1940):317-325.

[8]Olcott, H. S., and Fraenkel-Conrat, H.: Specific Group Reagents for Proteins. Chem. Rev., *41*, (1947):151-197.

[9]Green, H. H.: Weybridge P.P.D. Tuberculins. Vet. J., *102*, (1946):267-278.

[10]McIntosh, C. W., and Konst, H.: Comparison of Human and Bovine Type Tuberculin on Guinea Pigs Sensitized with Several Acid-Fast Microorganisms. Canad. J. Comp. Med. and Vet. Sci., *88*, (1947):93-101.

[11]Green, H. H.: Personal communication.

[12]Seibert, F. B., Pedersen, K. I., and Tiselius, A.: Molecular Weight, Electrochemical and Biological Properties of Tuberculin Protein and Polysaccharide Molecules. J. Expti. Med., *68*, (1938):413-438.

[13]Seibert, F. B.: The Chemical Composition of the Active Principle of Tuberculin. XI. An Improved and Simplified Method for Making a Standard Undenatured Tuberculin of any Strength and a Method of Chemical Assay. J. Biol. Chem., *78*, (1928):345-362.

Experimental Therapy of Brucellosis in Swine with Streptomycin and Sulfadiazine

L. M. HUTCHINGS, D.V.M., M.S., Ph.D., DORIS E. BUNNELL, B.S., M.S., and W. W. BAY, B.S., D.V.M.

Lafayette, Indiana

MANY REPORTS have appeared in the literature concerning the efficacy of combined streptomycin and sulfadiazine treatment against the various species of the genus Brucella.

LITERATURE CITED

Karlson and Feldman[1] reported on the bacteriostatic effect of streptomycin in low concentrations *in vitro* against a wide variety of bacteria, among those, *Brucella abortus*. Jones *et al.*[2] found that the chicken embryo could be protected against *Br. abortus* infection by treatment with streptomycin. Work of Spink *et al.*[3] showed that either streptomycin or sulfadiazine administered alone to chicken embryos previously inoculated with *Br. abortus* prolonged the lives of the embryos, but did not eradicate Brucella. However, when the two were injected simultaneously, the survival rate was greater and Brucella was absent from the tissues of a majority of embryos.

Live *et al.*[4] and Gilman and Lo Grow[5] reported on the effect of the treatment of experimental brucellosis in the guinea pig with streptomycin. From their reports, it seemed that if streptomycin therapy were continued long enough, it had a definite deterrent effect on the course of the disease.

Herrell and Nichols[6] reported on the treatment with streptomycin of 3 cases of undulant fever showing bacteriemia. They say, "While it was apparently possible to sterilize the blood stream temporarily from Brucella, the course of the disease and change in the patients' well-being were not permanently altered. In one case, however, the patient experienced a good response to streptomycin therapy following splenectomy."

Eisele and McCullough[7] and Spink *et al.*[8] have reported on the satisfactory use of combined therapy in cases of brucellosis in man. Eisele and McCullough stated, "No effect was noted on the clinical course, the fever or the septicemia (of the one case) when streptomycin and sulfadiazine were used separately in large doses. The simultaneous use of streptomycin and sulfadiazine resulted in prompt clinical and bacteriologic cure."

Since porcine brucellosis is apparently quite similar in its manifestations to brucellosis in man, and because of the relative ease of studying the course of the diseases in swine, it seemed advisable to use the pig as an experimental animal in an attempt to evaluate bacteriologically the combined sulfadiazine-streptomycin therapy as used in man.

EXPERIMENTAL PROCEDURE

Three experiments were conducted using a total of 30 pigs, weighing between 47.5 and 127.0 lb. Ten animals were used in each experiment, 5 serving as controls and 5 treated. Streptomycin in distilled water was administered intramuscularly at the rate of 2 Gm. per day, 1 Gm. at 8 a. m. and 1 Gm. at 4:00 p. m., in each series. In the first group, the treatment with streptomycin was continued for seven days. In the other two groups, the streptomycin therapy was prolonged for fourteen days. Sulfadiazine administration was started in each group simultaneously with streptomycin, but was continued for twenty-one days in all three groups. Each dose of sulfadiazine was suspended in approximately 100 cc. of distilled water and was given orally with a dose syringe. The starting dose of sulfadiazine was 1.5 gr. per pound of body weight and succeeding doses were 0.75 gr. per pound of body weight, twice a day at 8:00 a. m. and 4:00 p. m. The dose was varied somewhat in an attempt to keep the blood level of sulfadiazine around 5 mg. per 100 cc. of blood. The sulfadiazine-blood level was determined by the sulfonamide-blood level determination unit prepared by the LaMotte Company, a colorimetric determination.

Blood culture and serum-agglutination tests were conducted twice weekly for a short time prior to and during the course of the experiments. Treatment was started in the first two groups only after it had been shown that at least half of the animals were showing a Brucella bacteriemia. Treatment of group 1 was initiated thirty-one days after exposure; in group 2, twenty-three days postexposure.

Streptomycin for this study was furnished by courtesy of Merck and Co., Rahway, N. J.

Published as Journal Paper No. 458 of the Purdue University Agricultural Experiment Station.

These studies were supported in part by a grant from the Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture.

From the Department of Veterinary Science, Purdue University, Lafayette, Ind.

TABLE 1—Blood-Sulfadiazine Determinations for Group 1

	Milligrams of sulfadiazine per 100 cc. of blood								
Day	1		2			3		5	7
Hour	1:00 p.m.	4:00 p.m.	8:00 a.m.	1:30 p.m.	4:00 p.m.	8:00 a.m.	1:00 p.m.	8:00 a.m.	8:00 a.m.
3268	8.8	5.9	5.9	15.7	15.7	15.7	15.7	13.7	20.6
3226	7.8	4.9	8.8	6.9	15.7	15.7	17.7	44.1	44.1
3286	Clot	4.9	7.8	7.8	15.7	15.7	23.5	35.3	29.2
3219	7.8	5.9	11.8	11.8	17.6	17.7	27.4	13.7	20.6
3235	9.8	5.9	9.8	9.8	9.8	7.8	17.6	11.7	23.5

	Milligrams of sulfadiazine per 100 cc. of blood				
Day	8	11	13	15	17
Hour	8:00 a.m.	8:00 a.m.	1:00 p.m.	8:00 a.m.	8:00 a.m.
3268	29.6	23.5	5.9	Trace	Trace
3226	47.0	47.0	54.9	54.8	61.7
3286	31.4	29.2	37.4	11.8	8.8
3219	23.5	23.5	5.9	5.9	1.9
3235	13.7	20.6	17.6	Trace	Trace

TABLE 2—Blood-Sulfadiazine Determinations for Group 2

	Milligrams of sulfadiazine per 100 cc. of blood								
Day	3		4	6	7	11	14	18	21
Hour	9:00 a.m.	1:30 p.m.	8:30 a.m.	8:20 a.m.	8:30 a.m.	8:30 a.m.	8:30 a.m.	8:30 a.m.	8:30 a.m.
3302	2.0	2.8	5.9	1.8	3.9	4.0	2.0	7.8	9.0
3306	2.0	4.9	8.8	9.8	7.8	7.0	5.0	7.8	11.0
3312	2.0	8.8	3.9	2.0	2.9	6.0	4.0	7.8	9.0
3308	11.8	23.5	11.8	3.0	2.9	6.0	5.0	4.8	11.0
3304	2.0	2.9	4.9	20.0	39.2	5.0	6.0	2.0	1.0

TABLE 3—Blood-Sulfadiazine Determinations for Group 3

	Milligrams of sulfadiazine per 100 cc. of blood			
Day	2	7	11	18
Hour	8:30 a.m.	8:30 a.m.	8:30 a.m.	8:30 a.m.
11	2+	5	5.9	10
15	2+	5	5.9	10
29	2+	5	8.9	10
41	8	5	13.7	5
53	4	5	2.9	8

TABLE 4—Results of Combined Streptomycin and Sulfadiazine Therapy of Brucellosis in Swine, Experiment 1 (April 10, 1948, to June 10, 1948)

	Postexposure		Post treatment		Weight (lb.)		No. tissues positive for *Brucella suis* at autopsy
Hog (No.)	Maximum titer	No. positive blood cultures	Maximum titer	No. positive blood cultures	Initial	Final	
			Treated animals				
3268	100	3	50	0	109	110	3
3219	100	1	50	0	93	85	2
3226	100	2	100	0	120	94	1
3286	50	4	50	0	82	65	1
3235	50	3	50	0	117	126	2
			Untreated controls				
3247	100	3	50	0			2
3260	50	3	25	2			5
3208	50	3	100	1			9
3272	100	2	100	0			1
3233	50	1	50	0			3

*This animal died prior to termination of experiment.

The first group consisted of 10 gilts which were exposed by feeding approximately 10 Gm. of ground fetus and placenta from a naturally occurring abortion from which *Brucella suis* had been recovered previously. The aborted material had been kept in a deep freeze at 0 F. for one month. Exposure was repeated after six days by feeding approximately 10 cc. of a suspension of a 48-hour culture of *Br. suis*, the density of a No. 1 McFarland nephelometer, on a small amount of ground feed. These organisms had been recovered from the same abortion.

The second group of 4 males and 6 females was exposed *per os* to approximately 10 Gm. of the same aborted material that had been used in the first experiment. This material had been retained in the deep freeze as above. Four guinea pigs were injected subcutaneously with the fetus material to check on the virulence of the organisms. Blood serum-agglutination reactions became positive and *Br. suis* was isolated from livers and spleens of all 4 guinea pigs at autopsy. In addition to the aborted material, a suspension the density of a No. 1 McFarland nephelometer was prepared from a strain of *Br. suis* which had been isolated from 1 of the pigs on the first experiment and had been injected into and subsequently recovered from the guinea pigs. The culture used for exposure was a 48-hour culture which had been transferred once previously on tryptose agar (Difco). Each animal received 10 cc. of this suspension on a small amount of ground feed. Ten days later, the animals were exposed a third time to a strain of *Br. suis* which had been isolated from the uterus of a naturally infected sow ten days previously. One hundred cubic centimeters of a suspension the density of a No. 1 McFarland nephelometer was fed to the animals en masse on ground feed.

All of the swine were fasted for twelve hours before exposure *per os*.

The third group of animals, 8 males and 2 females, was naturally infected as determined by the serum-agglutination test. We were unable to demonstrate the presence of a bacteriemia in any of these 10 at the time of the first treatment.

The animals in all three groups were destroyed within ten days after the completion of each experiment and exhaustive bacteriologic examinations were made. The tissues and organs cultured were: popliteal, prefemoral, superficial inguinal, internal iliac, gastrohepatic, bronchial, cervical, mesenteric, retropharyngeal and mandibular lymph nodes, genital organs, kidneys, bladder, liver, spleen, lungs, heart, ham, shoulder and loin muscles, spinal cord, vertebrae, and rib marrow.

RESULTS

The sulfadiazine-blood levels for the three groups, as determined by the LaMotte unit at various times during treatment, are shown in tables 1, 2, and 3. Even though the doses of sulfadiazine were gauged by the weight of the animals, there was considerable variation in the concentration of the drug in the blood of the different animals. This might indicate individual variation in the ability of the body to handle the substance. Considerable variations of the dosage were made to regulate the blood level.

Table 4 shows the detailed results of the treatment of the first group of 5 animals and the controls for the same experiment. In the treated animals, the bacteriemia ceased immediately following the initiation of treatment, whereas in the control animals, it persisted in 2 cases (pigs 3260 and 3208) for an additional seven days.

Three isolations of *Br. suis* were made from pig 3268, two each from pig 3219 and pig 3235, one from pig 3286, and none from

TABLE 5—Results of Combined Streptomycin and Sulfadiazine Therapy of Brucellosis in Swine, Experiment 2 (July 14, 1948, to August 30, 1948)

Hog (No.)	Postexposure		Post treatment		Weight (lb.)		No. tissues positive for *Brucella suis* at autopsy
	Maximum titer	No. positive blood cultures	Maximum titer	No. positive blood cultures	Initial	Final	
Treated animals							
3302	50	2	100	0	96	118	0
3295	100	4	100	0	107	85	0
3306	50	3	25	0	127	152	2
3308	50	1	50	0	96	116	0
3312	50	4	100	0	108	124	1
Untreated controls							
3310	50	3	100	1	100	124	2
3314	100	1	100	0	100	140	1
3316	100	2	100	4	126	148	7
3318	200	4	400	2	93	122	7
3320	100	3	50	1	134	139	6

pig 3226, making an average of 1.6 isolations per animal in the treated animals. In the controls, *Br. suis* was isolated from nine sources from pig 3208, five from pig 3260, three from pig 3233, two from pig 3247, one from pig 3272, for an average of 4.0 isolations per animal.

In table 5 are set forth the detailed observations on group 2. Again, it will be seen that bacteriemia ceased abruptly following the initiation of treatment, while in the controls, it continued for as long as three weeks (pigs 3310, 3316, 3318, and 3320).

Isolations of *Br. suis* were made from only 2 of the treated animals at autopsy, two isolations from pig 3306, one from pig 3312, for an average of 0.6 isolations per animal. In the untreated controls, *Br. suis* was isolated from seven sources from pigs 3316 and 3318, six sources from pig 3320, two sources from pig 3310, and one isolation was made from pig 3314, making an average of 4.6 isolations per animal.

The detailed results of experiment 3 are shown in table 6. These animals were naturally infected, as judged by the agglutination reactions. The only positive blood culture observed during the course of this trial was obtained from pig 33, one of the control animals on the day treatment was started.

In this group, *Br. suis* was isolated once from pig 41, twice from pig 53, four times from pig 11, and five times from pig 29. No isolations were made from pig 15. This makes an average of 2.4 isolations per treated pig. From the controls, *Br. suis* was isolated from 15 sources from pig 31, 11 sources from pig 33, ten sources from pig 49, six sources from pig 19, and five sources from pig 47, making an average of 9.4 isolations per animal.

The treatment had no observable effect on the agglutination response of any of the animals. One animal in each of the treated groups showed apparent, extreme toxic reactions to the treatment. These pigs were in such bad condition that the treatment was discontinued entirely. Pig 3226 (table 4) died before the end of the experiment, apparently as a result of the treatment. The treated pigs, on the whole, were in poorer condition than the controls, as evidenced by appearance, appetite, and weight. Unfortunately, initial weights on the untreated controls of group 1 were not obtained. When observations on the first treatment indicated the loss of weight in the treated animals, this information was obtained for the last two groups. Although the sulfadiazine-blood levels of group 1 were extremely high, the blood levels of groups 2 and 3 were nearer the desired concentration. The condition of the treated pigs in all three lots was similar.

SUMMARY AND CONCLUSIONS

In this report are included the records of experiments with the treatment of *Brucella suis*-infected pigs with streptomycin and sulfadiazine. Three experiments were conducted, each using 10 pigs, 5 treated and 5 untreated controls, making a total of 30 animals. Two of the groups were experimentally infected with *Br. suis per os*, while the third was naturally infected, as determined by serum-agglutination reactions.

In all cases where blood culture was positive before treatment, the bacteriemia

TABLE 6—Results of Combined Streptomycin and Sulfadiazine Therapy of Brucellosis in Swine, Experiment 3 (October 15, 1948, to December 1, 1948)

Hog (No.)	Postexposure		Post treatment		Weight (lb.)		No. tissues positive for *Brucella suis* at autopsy
	Maximum titer	No. positive blood cultures	Maximum titer	No. positive blood cultures	Initial	Final	
Treated animals							
41	400	0	400	0	82	59	1
29	100	0	400	0	71	75	5
11	400	0	400	0	56	41	4
53	100	0	200	0	51	65	2
15	400	0	400	0	72	84	0
Untreated controls							
33	100	1	100	0	62	80	11
49	400	0	400	0	48	55	10
47	400	0	400	0	89	91	5
31	400	0	400	0	67	94	15
19	200	0	200	0	78	97	6

ceased following initiation of treatment, whereas in the controls, the bacteriemia persisted for varying lengths of time, as long as three weeks in 1 case.

Treatment seemed to have a definite effect in dimunition of numbers of isolations made at autopsy. It must be emphasized, however, that *Br. suis* was isolated from 10 of the 15 treated animals at autopsy. Thus, while the circulating blood was apparently freed of Brucella organisms, there still remained foci of Brucella throughout the body.

The condition of the treated animals on the whole was much poorer than that of the controls. As a rule, weight losses occurred, or weight was barely maintained in the treated groups, while the control groups gained normally. One animal of each of the treated groups showed extreme symptoms of toxic reaction apparently as a result of the treatment. These results suggest that the dosages given were at or near the maximum that can be tolerated by pigs.

References

[1]Karlson, A. G., and Feldman, W. H.: Streptomycin in Experimental Infections and Its Possible Use in Veterinary Therapeutics. J.A.V.M.A., *110*, (1947) :63-70.

[2]Jones, Doris, Metzger, H. J., Schatz, Albert, and Waksman, S. A.: Control of Gram-Negative Bacteria in Experimental Animals by Streptomycin. Science, *96*, (1944) :103-105.

[3]Spink, W. W., Hall, W. H., Shaffer, J. M., and Braude, A. I.: Human Brucellosis. J.A.M.A., *136*, (1947) :382-387.

[4]Live, I., Sperling, F. G., and Stubbs, E. L.: Effect of Streptomycin on Experimental Brucellosis in Guinea Pigs. Am. J. M. Sc., *21*, (1946) :267-272.

[5]Silman, H. L., and Le Grow, W. R.: Streptomycin in the Treatment of Experimental Brucellosis of Guinea Pigs. Am. J. Vet. Res., *8*, (1947) :192-195.

[6]Herrell, W. E., and Nichols, D. R.: The Clinical Use of Streptomycin: A Study of Forty-Five Cases. Proc. Staff Meet. Mayo Clinic, *20*, (1945) :449-462.

[7]Eisele, C. W., and McCullough, N. B.: Combined Streptomycin and Sulfadiazine Treatment in Brucellosis. J.A.M.A., *135*, (1947) :1053-1059.

[8]Spink, W. W. Hall, W. R., Shaffer, J. M., and Braude, A. I.: Human Brucellosis, Its Specific Treatment with a Combination of Streptomycin and Sulfadiazine. J.A.M.A., *136*, (1948) :382-387.

Studies on the Hematology of the Thoroughbred Horse
II. Weanlings

M. F. HANSEN, Ph.D., A. C. TODD, Ph.D., G. W. KELLEY, B.S.,
M. CAWEIN, B.A., and W. R. McGEE, D.V.M.

Lexington, Kentucky

HANSEN *et al.*[1] in a study of 65 Thoroughbred mares in foal reported values for the erythrocyte and leucocyte counts, hemoglobin concentration, packed volume of erythrocytes, mean corpuscular volume, mean corpuscular hemoglobin concentration, and differential counts. The present study details the results of the above blood values for a population of 70 healthy Thoroughbred weanlings from six different Thoroughbred farms in central Kentucky.

A population of 70 weanlings is a more representative number upon which to base studies of blood values than the 22 weanlings used by MacLeod *et al.*[2] The present paper also extends their work by including data on the packed volume of erythrocytes, mean corpuscular volume, and mean corpuscular hemoglobin concentration, together with statistical analyses and measurements of reliability of the results. A statistical test was made to determine any differences in the mean erythrocyte count and mean hemoglobin content between Thoroughbred weanling colts and fillies.

METHODS

The same methods were used in this study as were detailed in the first study in the series on the hematology of the Thoroughbred horse by Hansen *et al.*[1] Blood samples were collected under as nearly indentical conditions as possible with respect to time of day, season of year, time of feeding, watering, length of time in stall before bleeding, and the degree of excitement before the blood sample was obtained. Accepted laboratory procedures, equipment, and the same measurements of laboratory error as detailed in the first paper of this series were used.

Statistical computations were made to obtain arithmetic averages, standard deviations, and standard errors of each blood value. Curves representing normal probability were fitted to each distribution and their "goodness of fit" was tested by the use of *chi* square. A measure of skewness utilizing the mean and median was used.

TABLE I—Blood Picture of 70 Healthy Thoroughbred Weanlings in Central Kentucky

Measure	Erythrocytes 10^6/cmm.	Hemoglobin Gm./100 ml.	Packed volume of erythrocytes (%)	Leucocytes 10^3/cmm.	Mean corpuscular volume (cubic microns)	Mean corpuscular hemoglobin concentration (%)
Range	9.60-12.81	10.2-13.7	30.2-42.0	7.62-18.57	29.5-36.2	30.1-34.6
Median	11.25	11.96	37.06	13.50	32.50	32.70
Arithmetic mean and s.e.*	11.24 ± 0.097	11.97 ± 0.090	36.80 ± 0.318	13.64 ± 0.246	32.72 ± 0.187	32.53 ± 0.138
Standard deviation	0.82 ± 0.068	0.75 ± 0.064	2.66 ± 0.225	2.06 ± 0.174	1.56 ± 0.132	1.15 ± 0.097

	Leucocytes				
	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
Range	20.5-73.5	22.5-73.0	0.5-7.6	0.5-7.0	0-1.0
Mean and s.e.	45.0 ± 1.290	49.7 ± 1.481	2.72 ± 0.233	2.3 ± 0.203	<0.1
Standard deviation	10.79	12.39	1.87	1.70	....

*Standard error.

RESULTS AND DISCUSSION

Ranges, medians, arithmetic means, and standard deviations, as well as standard

The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the director.

From the Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington.

Dr. McGee is a veterinarian with Hagyard and Hagyard, Lexington.

The authors express their appreciation to Dr. Dana G. Card, Department of Markets and Rural Finance, University of Kentucky, for his assistance in the statistical procedures.

errors of the means and standard deviations for each of the six blood values, are given in table 1.

The arithmetic means with their standard errors appear to be quite reliable, even though the ranges indicated considerable variation from weanling to weanling (table 1). In most cases, the arithmetic means have a two to one chance of being correct within 1.0 per cent of the reported mean. The only exception is the mean value for the number of leucocytes which has a two to one chance of being correct within about 2.0 per cent. The leucocyte counts varied considerably from weanling to weanling, as indicated by the range. Such variations in the leucocyte counts are to be expected in view of the fact that conditions causing mild leucopenia or leucocytosis are frequently not overtly expressed in the general physical condition of an animal.

The mean count of 11,240,000 erythrocytes per cubic millimeter reported in this paper appears to agree fairly closely with the mean count of 11,900,000 erythrocytes per cubic millimeter reported by MacLeod *et al.*[2] No accurate determination of any statistically significant difference between means can be made because MacLeod did not give the standard error of the mean value of erythrocytes in his study. Our mean value of 11.97 Gm. of hemoglobin per 100 ml. of blood is somewhat lower than their reported mean of 13.0 Gm. This apparent disagreement in the mean hemoglobin content is probably due to the use of different methods of hemoglobin determination. MacLeod used the Sahli method for hemoglobin determination while we used the Fisher electrohemometer. The latter method of determining hemoglobin concentration eliminated the personal equation of matching a color standard. The two studies are in perfect agreement in reporting a mean number of 13,600 leucocytes per cubic millimeter.

There has been no report in the literature on the values for packed volume of erythrocytes, mean corpuscular volume, or mean corpuscular hemoglobin concentration for the Thoroughbred weanling. The following mean values and their standard errors are recorded for the Thoroughbred weanling: packed volume of erythrocytes, 36.80 ± 0.318 per cent; mean corpuscular volume, 32.72 ± 0.187 cubic microns; and a mean corpuscular hemoglobin concentration of 32.53 ± 0.138 per cent.

The distribution of measurements from these weanlings has been tested for normality and appears not to be more skewed than might happen as a result of sampling error, although the mean corpuscular volume and mean corpuscular hemoglobin concentration were more noticeably skewed than the others.*

The distribution of the various types of leucocytes is given in table 1. Our study, as did the study by MacLeod, showed a greater mean percentage of lymphocytes than neutrophils, but the present study shows a lower mean percentage of monocytes and eosinophils than did MacLeod's.

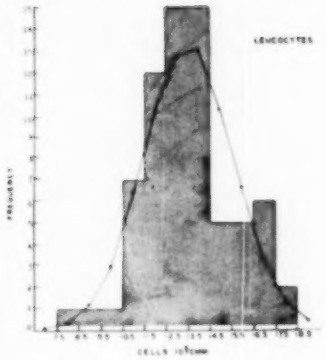
A comparison of the mean erythrocyte count and hemoglobin content was made for the 37 weanling colts and 33 weanling fillies used in this study. The fillies had a mean value of 11,280,000 erythrocytes per cubic millimeter and 12.09 Gm. of hemoglobin per 100 ml. of blood; the colts had a mean value of 11,200,000 erythrocytes per cubic millimeter and 11.87 Gm. of hemoglobin per 100 ml. of blood. Even though the fillies showed slightly higher mean values for the number of erythrocytes and hemoglobin content, the differences between the mean values for fillies and colts were not statistically significant.

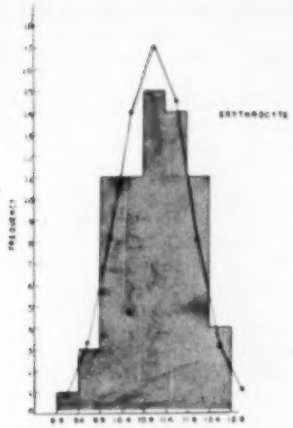
In order to determine what influence the degree of parasitism might have on the reported blood values, the egg counts, erythrocyte counts, hemoglobin, and leucocyte counts of weanlings on two farms were compared. The weanlings at one farm showed the following averages in eggs per gram of feces: ascarids, 58.8; strongyles,

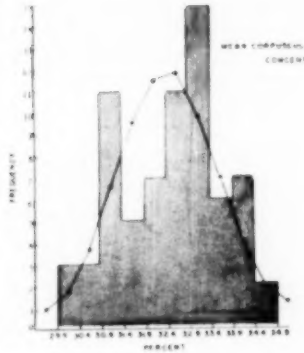
*All of the blood values determined in this study were fitted to a curve of normal distribution. In the case of red and white blood cell counts, a curve of Poisson distribution may have been more appropriate.

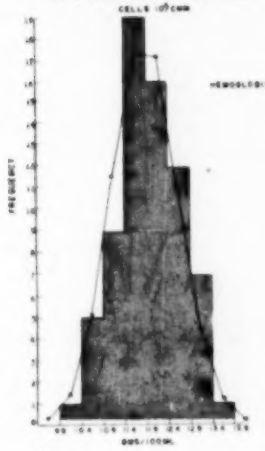
Legend for Illustration on Opposite Page

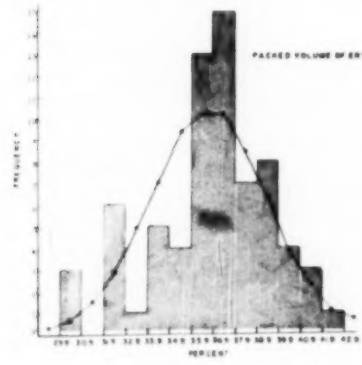
Fig. 1.—The histograms represent the observed frequency distributions and the polygons represent the theoretic distributions for the erythrocytes in millions per cubic millimeter, hemoglobin content, leucocytes in thousands per cubic millimeter, mean corpuscular volume, and the mean corpuscular hemoglobin concentration.

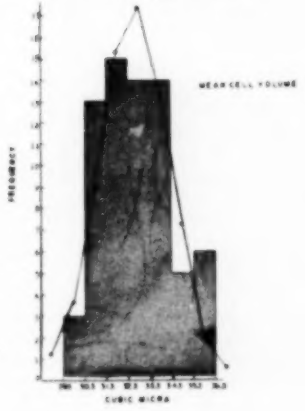

LEUCOCYTES
FREQUENCY


ERYTHROCYTE
FREQUENCY


FREQUENCY


HEMOGLOBIN
FREQUENCY


PACKED VOLUME OF ERYTHROCYTES
FREQUENCY


MEAN CELL VOLUME
FREQUENCY

11.28; and Strongyloides spp., 6.8. The average blood values were: erythrocytes, 11,600,000 per cubic millimeter; hemoglobin, 12.3 Gm. per 100 ml.; and leucocytes, 14,600 per cubic millimeter. The weanlings at the second farm showed the following averages in eggs per gram of feces: ascarids, 38.0; strongyles, 16.0; and Strongyloides spp., 2.0. The average blood values were: erythrocytes, 11,400,000 per cubic millimeter; hemoglobin, 11.9 Gm. per 100 ml.; and leucocytes, 13,400 per cubic millimeter. The degree of parasitism in the weanlings at these two farms is representative of all weanlings used in this study. The weanlings at both farms showed about the same degree of parasitism; the blood values were likewise very similar. It is probable that weanlings which have never been parasitized by these helminths would show significantly different blood values than reported in this study; however, such animals are not to be found under field conditions. This study has dealt, therefore, with the blood values of a population of lightly to moderately parasitized weanlings.

SUMMARY

1) A hematologic study of a population of 70 Thoroughbred weanlings revealed the following mean values with their standard errors: erythrocytes per cubic millimeter, 11.24 ± 0.097 million; hemoglobin grams per 100 ml. of blood, 11.97 ± 0.090; leucocytes per cubic millimeter, 13.64 ± 0.246 thousand; packed volume of erythrocytes, 36.80 ± 0.318 per cent; mean corpuscular volume, 32.72 ± 0.187 cubic microns; and a mean corpuscular hemoglobin concentration of 32.53 ± 0.138 per cent.

2) The differential leucocyte counts showed the following mean percentages and standard errors: neutrophils, 45.0 ± 1.290; lymphocytes, 49.7 ± 1.481; monocytes, 2.72 ± 0.233; eosinophils, 2.3 ± 0.203; and basophils less than 0.1.

3) Results of *chi*-square tests of agreement between the observed distributions and theoretic distributions of blood values according to the normal curve, together with measurement of skewness, indicate that the normal curve of error is applicable to the distributions, with the possible exception of the mean corpuscular volume and mean corpuscular hemoglobin concentration.

4) Even though the weanling fillies showed slightly higher mean values for the number of erythrocytes per cubic millimeter and hemoglobin grams per 100 ml. of blood than did the weanling colts, the differences between the mean values were not statistically significant.

References

[1]Hansen, M. F., Todd, A. C., Kelley, G. W., and Hull, F. E.: Studies on the Hematology of the Thoroughbred Horse. I. Mares in Foal. Am. J. Vet. Res., *11*: (1950): 286-300.

[2]MacLeod, J., Ponder, E., Aitkin, G. J., and Brown, R. S.: The Blood Picture of the Thoroughbred Horse. Cornell Vet., 37, (1947): 305-313.

Studies on the Hematology of the Thoroughbred Horse
III. Stallions

M. F. HANSEN, Ph.D., A. C. TODD, Ph.D., M. CAWEIN, B. A., and W. R. McGEE, D.V.M.

Lexington, Kentucky

IN STUDIES of 65 healthy Thoroughbred mares in foal and 70 Thoroughbred weanlings, Hansen *et al.*[1, 2] have reported the values for the erythrocyte and leucocyte counts, hemoglobin content, packed volume of erythrocytes, mean corpuscular volume, mean corpuscular hemoglobin concentration, and differential counts. There is no information, as such, in the literature on these blood values for healthy Thoroughbred stallions. MacLeod *et al.*[3] reported the mean values for erythrocyte and leucocyte counts, hemoglobin content, and differential counts on 12 Thoroughbred horses which they classified as "Older horses (other than mares)." Such a classification could include geldings, stallions, and young fillies, so that one cannot assign MacLeod's reported mean values exclusively to stallions.

The present study details the average blood values for 36 healthy Thoroughbred stallions from six different farms in central Kentucky.

METHODS

The methods used in this study are given in the first study of this series.[1]

This study was conducted on the stallions before the breeding season started. Blood samples were collected from 12 of the stallions in the latter part of November, the remaining 24 stallions were bled in the early part of January. All but four of the blood samples were collected between 8 and 9 a. m. Four of the samples could be collected only at 2 p. m. because of a morning exercise schedule used for the stallions on one of the farms visited. All the stallions had been resting in their stalls for two or more hours before the blood samples were collected. Every effort was made to obtain blood samples without exciting the stallions; however, 2 of the 36 stallions were very excited. Consequently, their erythrocyte counts, hemoglobin, and packed volume of erythrocytes are the extreme highs of the ranges reported for these blood values.

Accepted laboratory procedures, equipment, and the same measurements of laboratory error as detailed in the first paper of this series were used. Statistical computations were

TABLE I—Blood Picture of 36 Healthy Thoroughbred Stallions in Central Kentucky

Measure	Erythrocytes (10^6/cmm.)	Hemoglobin (Gm./100 ml.)	Packed volume of erythrocytes (%)	Leucocytes (10^3/cmm.)	Mean corpuscular volume (cubic microns)	Mean corpuscular hemoglobin concentration (%)
Range	8.33-13.30	12.0-18.3	38.0-58.5	5.05-11.90	39.1-46.4	30.1-33.6
Median	10.71	14.7	46.9	7.95	43.2	31.2
Arithmetic mean and s.e.*	10.81 ± 0.174	14.69 ± 0.261	46.78 ± 0.744	8.27 ± 0.245	43.16 ± 0.290	31.33 ± 0.121
Standard deviation	1.04 ± 0.122	1.56 ± 0.183	4.64 ± 0.546	1.47 ± 0.173	1.74 ± 0.205	0.726 ± 0.085

	Leucocytes				
Measure	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
Range	35.0-65.0	30.0-57.5	0.5-7.0	0.5-7.0	0.0-2.0
Mean and s.e.	50.75 ± 1.160	43.75 ± 1.162	2.0 ± 0.245	4.0 ± 0.277	0.5 ± 0.077
Standard deviation	6.82	6.97	1.47	1.66	0.465

*Standard error.

From the Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington.
Dr. McGee is a veterinarian with Hagyard and Hagyard, Lexington.
The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the director.

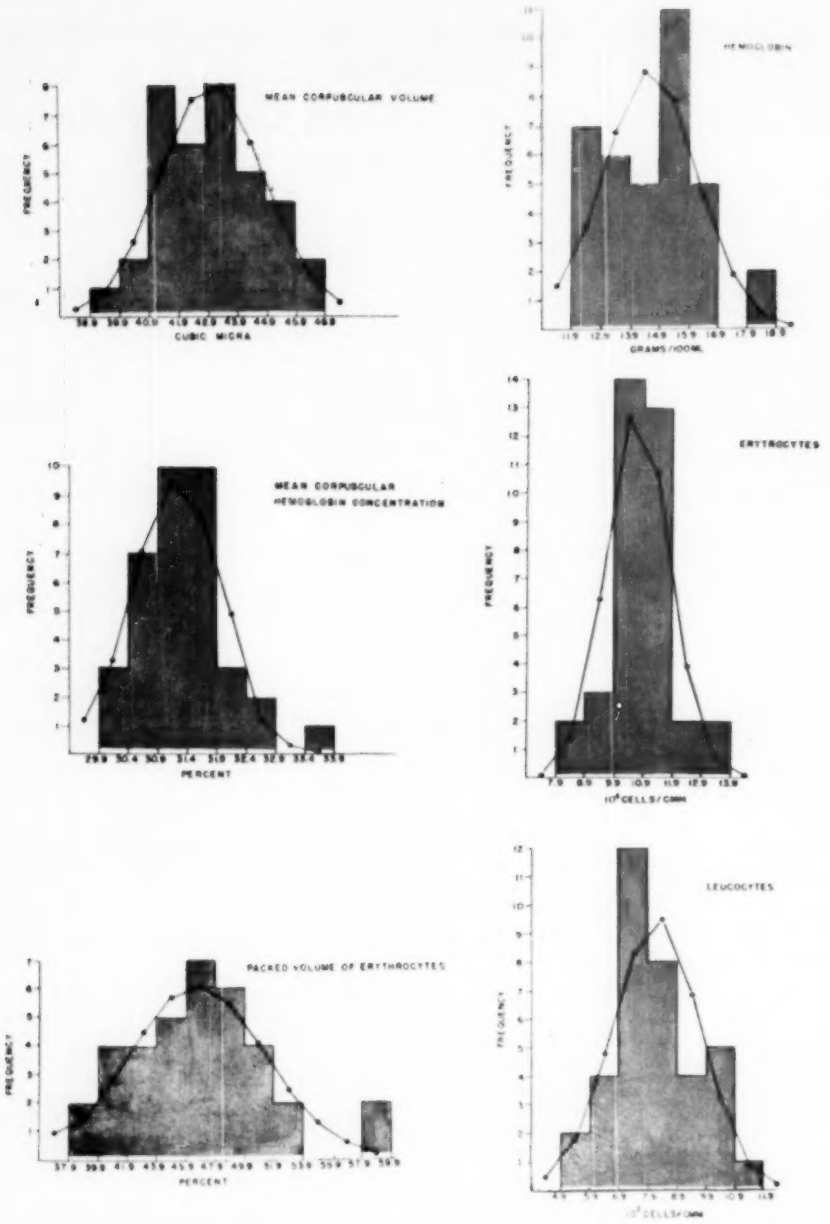


Fig. 1—The histograms represent the observed frequency distributions and the polygons represent the theoretic distributions for the mean corpuscular hemoglobin concentrations, mean cell volume, hemoglobin content, leucocytes in thousands per cubic millimeter, erythrocytes in millions per cubic millimeter, and the packed volume of erythrocytes.

used to obtain arithmetic averages, standard deviations, and standard errors of each blood value. Curves representing normal probability were fitted to each distribution and their "goodness of fit" was tested by use of *chi* square. A measure of skewness utilizing the mean and median was used.

RESULTS AND DISCUSSION

In table 1 are given the values for the ranges, medians, arithmetic means, and standard deviations, as well as standard errors of the means and standard deviations for each of the six blood values of 36 healthy Thoroughbred stallions.

A comparison of the arithmetic means with their standard errors for the six blood values suggests that the averages are quite reliable, even though these blood values varied considerably from stallion to stallion, as shown by the ranges and standard deviations. In most cases, the arithmetic means of the blood values have a two to one chance of being correct within about 3.0 per cent. Similar variations for the six blood values from individual to individual have been reported for mares in foal as well as for weanlings.[1, 2]

The differential leucocyte counts varied appreciably from stallion to stallion. The number and type of leucocytes to be found circulating in the blood at any one time varies considerably under the influence of numerous conditions which are difficult to control, e.g., a small infected scratch would not be noticed when the stallion was inspected for general physical condition; a subsequent leucocyte and differential count might well reveal a mild leucocytosis and neutrophilia. Furthermore, difficulty in obtaining a uniform distribution of the various types of leucocytes in the differential film and the counting of only a small part of the total number of leucocytes present introduce an appreciable amount of technical error.

The distributions of four of the blood values appear not to be more skewed than might result from error of sampling (fig. 1). The hemoglobin and mean corpuscular hemoglobin concentration, however, showed approximate significance levels of agreement with a normal curve at only the 15 and 40 per cent levels, respectively, for *chi*-square probabilities.

In the two previous studies on the hematology of the Thoroughbred horse, Hansen *et al.*[1, 2] found that the parasite burdens observed in the horses in central Kentucky did not influence the blood picture to any appreciable extent. Fecal egg counts on 6 stallions at one of the farms visited revealed a negligible number of helminths. The total eggs per gram of feces ranged from 0 to 144, 2 of the stallions having negative egg counts. Strongyles were the predominant type of parasite found. Similar egg counts could be expected for stallions on the other farms visited.

SUMMARY

1) The following mean blood values with their standard errors are recorded from a population of 36 Thoroughbred stallions: erythrocytes per cubic millimeter, 10.81 ± 0.174 million; hemoglobin, Gm. per 100 ml. of blood, 14.69 ± 0.261; leucocytes per cubic millimeter, 8.27 ± 0.245 thousand; packed volume of erythrocytes, 46.78 ± 0.744 per cent; mean corpuscular volume, 43.16 ± 0.290 cubic microns; and a mean corpuscular hemoglobin concentration of 31.33 ± 0.121 per cent.

2) The differential leucocyte counts showed the following mean percentages and standard errors: neutrophils, 50.75 ± 1.160; lymphocytes, 43.75 ± 1.162; monocytes, 2.0 ± 0.245; eosinophils, 4.0 ± 0.277; and basophils, 0.5 ± 0.077.

3) Results of the *chi*-square tests of agreement between observed distributions and theoretic distributions of blood values according to the normal curve indicate that the normal curve of error is applicable to the distributions, with the possible exception of the hemoglobin content and mean corpuscular hemoglobin concentration.

References

[1]Hansen, M. F., Todd, A. C., Kelley, G. W., and Holl, F. E.: Studies on the Hematology of the Thoroughbred Horse. I. Mares in Foal. Am. J. Vet. Res., *11*, (1950) :286-300.

[2]Hansen, M. F., Todd, A. C., Kelley, G. W., Cawein, M., and McGee, W. R.: Studies on the Hematology of the Thoroughbred Horse. II. Weanlings. Am. J. Vet. Res., *11*, (1950) : 393-396.

[3]MacLeod, J., Ponder, E., Aitken, G. J., and Brown, E. B.: The Blood Picture of the Thoroughbred Horse. Cornell Vet., *37*, (1947) :305-313.

A Quantitative Investigation of Some Mineral Components of Wheat Plants

MARGRET STUART, M.A., F. G. HARBAUGH, D.V.M., and JOE DENNIS, Ph.D.

Lubbock, Texas

CATTLE GRAZING wheat occasionally exhibit symptoms of hypersensitivity and tetany or the coma and paralysis resembling parturient paresis. The inference has been that the development of such conditions is attributable to the diet which the animals consume. It has been shown that certain changes occur in the blood picture of the afflicted animals.[1, 2] The most consistent change which has been reported is in the blood calcium content. The frequent similarity of these symptoms to those of "grass staggers," as the latter have been reported by Sjollema,[3] has led some workers to suppose that "wheat poisoning" also may be accompanied by a low blood magnesium content. McMillen and Langham seem to have answered this negatively.[1] There is some indication that high blood potassium may be a causative factor in the extreme hypersensitiveness which is occasionally encountered.[4]

If the symptoms are the result of grazing wheat, it seems logical to look to the wheat for some deficiency or for an excess of some one or more of the metallic ions which have been mentioned as playing a role in the development of the condition. It is possible that there are factors which might influence adversely the absorption of certain ions. We conducted a series of analyses on the calcium, magnesium, phosphorus, potassium, sodium, and manganese contents of the aerial portion of wheat plants. Similar analyses were run on those portions of these elements which are extractable with water and on the amount of each element left in the residue from such water extractions. It was thought that if any of the calcium or other minerals is in wheat in an insoluble form this might not be available to the animal because of an inability to absorb it.

The data in this paper have been taken from a thesis presented to the Division of Graduate Studies by Miss Stuart in partial fulfillment of the requirements for the degree of master of arts.

From the Department of Chemistry (Stuart and Dennis); and the Department of Animal Husbandry (Harbaugh); Texas Technological College, Lubbock.

PROCEDURE

The wheat plants which were subjected to analysis were gathered during the fall and spring of 1947-1948 and of 1948-1949. Some samples were chosen at random from the college farm, but the majority came from one or the other of two areas which were planted in a location convenient for irrigation. The samples were taken by breaking the plants about ½ to 1 in. above the ground. With one exception, they were gathered before 10 a. m. The plants were dipped in distilled water to remove the adhering soil and then centrifuged in a basket for three to five minutes to remove the excess moisture. They were then placed on clean towels on a large table, winnowed, and allowed to remain in the air for several minutes. Excess moisture was completely removed by this procedure. Three samples were then selected, one for the moisture determination, one for the extraction, and one for total mineral analysis.

The moisture content was run in the accepted manner[5] in tared aluminum vessels, drying for five hours at 100 to 102 C. The sample for extraction (usually about 400 Gm.) was placed in Mason jars, sealed, and kept overnight in a refrigerator at 14 F. The frozen sample was ground in a meat chopper, distilled water was added, and the mixture beaten in a Waring blendor. The mixture was strained through an ordinary kitchen sieve, and the solid material pressed at 10,000 lb. The fibers were subjected to the extraction process again. Earlier samples were extracted three times, and later ones, four times. Preliminary investigations showed that three such extractions removed the water-soluble material. The extract was centrifuged, filtered, and the filtrate evaporated to dryness on a hot plate. The solid residue thus obtained was transferred quantitatively to platinum dishes and ashed in the same manner as the whole plants. The fibrous residues from the extraction process were dried and ashed.

The plants to be used for the determination of total mineral content were dried at 100 to 102 C. for five hours. The sample was ground and stored in glass containers.

The analytic methods employed were essentially those of the Association of Official Agricultural Chemists.[6]

RESULTS AND DISCUSSION

Table 1 shows the figures obtained on the analysis of the entire aerial portion of the wheat plants and on the residues from the extraction of such plants. The figures shown on the extractable portions of the various elements were obtained by difference. The analyses for the various elements extractable from the wheat plants were run in all cases. The evaporations were accomplished in aluminum pans in order that silica figures might be reasonably accurate. It was then thought necessary to remove the aluminum, iron, etc., by precipitation with ammonia; however, it was found that a large part of the calcium and magnesium of the extracts was lost in this manner.* As a consequence, the results on only four of the samples, on which the evaporations were accomplished in glass and the ammonia treatment omitted, are considered significant.[7]

Examination of table 2 reveals that the minerals supplied by 140 lb. of the aerial portion of wheat plants, and also the extractable minerals, are sufficient in amount to supply the daily requirements of a 1,000-lb. beef cow. We do not have figures indicating the requirements for sodium, potassium, and manganese. It is of interest to note that the amount of soluble potassium which might be ingested in wheat represents approximately 1,092 Gm. of potassium chloride or about 1 Gm. per pound of body weight for a 1,000-lb. cow. Administration of such amounts of potassium chloride to cattle by stomach tube has been shown to result in symptoms resembling those of wheat poisoning.[4] The production of such symptoms was not found possible when the potassium chloride was fed to cattle. Pearson, Gray, and Reiser[8] found that feeding of potassium bicarbonate to ewes did not cause an increase in the plasma potassium. We believe that this failure to obtain an increase was due to the fact that a fairly long period, about four to six hours,[9] elapsed between the time of ingestion of the salt and the time of blood sampling. In the work of Dennis and Harbaugh, the time between the consumption of the morning portion of the potassium chloride and the blood sampling was about two to three hours. The rapid removal of potassium from the plasma[10] makes it improbable that an elevation in plasma potassium will be obtained unless the blood sampling is done during the period of rapid absorption into the blood. We are at present inclined to believe that the potassium content of the wheat plants is not the direct causative factor of the symptoms of wheat poisoning. It is, of course, not impossible that potassium reservoirs are built up in muscle, liver, etc., and that under certain conditions these reservoirs may release potassium with the production of the symptoms. The ratio of soluble calcium to soluble phosphorus is such that maximum conditions for absorption of calcium are not furnished by wheat. If the total calcium and phosphorus content of wheat plants represents that available to the animals, then this is not true.

The high ratio of magnesium to calcium does not facilitate absorption of calcium.[11] Magnesium, in the form of magnesium sulfate, in considerably less amounts than these, has been shown to result in a negative calcium balance in a feeding experiment with cows.[12] Much of the magnesium in the wheat is, of course, in the form of chlorophyll, and it may not exist in the ionic condition at any time in the digestive tract.

The figures on manganese content given in table 1 appear to be within the range of values found by other workers in other types of grasses.[13] Rats, when fed diets containing manganese in excess of 50 parts per million, have been shown to develop negative calcium balance.[14] The supplementation of the diets of cattle with manganese sulfate also results in disturbances of calcium metabolism.[15] The information on the manganese requirements and tolerances of cattle is not sufficiently extensive to predict the results of the consumption of manganese at the levels found in wheat. Experiments are being conducted along these lines. In connection with the manganese content of other grasses, it is well to remember the similarity of the symptoms of grass staggers and wheat poisoning.

The ratio between the potassium and sodium in wheat plants is 4.24 (table 2). This ratio is far more favorable than a similar one calculated from the figures given by Morrison on other green roughages.[16] This calculated average ratio for six different plants is 11.71. The figures indi-

*We are indebted to Dr. W. M. Craig and his students for the spectrographic analyses which revealed the presence of calcium and magnesium in the R_2O_3 precipitates.

TABLE 1—Showing the Analyses of Wheat Plants*

Date of Sample	Moisture %	SiO_2 %			Ca %			Mg %		
	W	W**	E**	R**	W	E	R	W	E	R
12-5-47	80.15	0.483	0.081	0.402	0.0773	0.0596	0.0177	0.0676	0.0568	0.0108
1-2-48	70.53	0.655	0.124	0.531	0.114	0.0853	0.0287	0.0892	0.0768	0.0124
2-19-48	71.35	0.923		0.997	0.144	0.0937	0.0503	0.0915	0.0684	0.0231
3-1-48	79.22	0.586	0.070	0.516	0.0955	0.0671	0.0284	0.0525	0.0387	0.0138
3-8-48	76.88	0.603	0.098	0.505	0.118	0.0878	0.0302	0.0736	0.0577	0.0159
3-15-48	77.47	0.586	0.090	0.496	0.120	0.0836	0.0364	0.0800	0.0614	0.0186
3-22-48	77.62	0.606	0.062	0.544	0.118	0.0862	0.0318	0.0678	0.0501	0.0177
3-29-48	77.70	0.484	0.029	0.455	0.0859	0.0608	0.0251	0.0502	0.0373	0.0129
4-5-48	77.24	0.572	0.026	0.546	0.0870	0.0591	0.0279	0.0548	0.0364	0.0184
4-12-48	71.66	0.655	0.109	0.546	0.107	0.0755	0.0315	0.0700	0.0526	0.0174
10-7-48	78.29	0.931	0.172	0.759	0.0880	0.0656	0.0224	0.0703	0.0571	0.0132
10-21-48	82.93	0.814	0.059	0.755	0.0731	0.0430	0.0301	0.0397	0.0228	0.0169
11-4-48	82.01	0.765	0.092	0.673	0.0841	0.0540	0.0301	0.0606	0.0445	0.0161
11-18-48	78.64	0.746	0.090	0.656	0.110	0.0818	0.0282	0.0699	0.0531	0.0168
12-9-48	73.88	0.946	0.121	0.825	0.116	0.0767	0.0393	0.0860	0.0670	0.0190
12-22-48	72.40	0.848	0.205	0.643	0.110	0.0754	0.0346	0.0802	0.0587	0.0215
2-28-48	77.09	0.571	0.108	0.463	0.0917	0.0639	0.0278	0.0566	0.0405	0.0161
3-14-48	80.31	0.676	0.172	0.504	0.0816	0.0590	0.0226	0.0473	0.0351	0.0122
3-28-48	78.33	0.832	0.199	0.633	0.0830	0.0615	0.0215	0.0522	0.0402	0.0120
4-11-48	78.60	0.979	0.048	0.931	0.0765	0.0501	0.0264	0.0478	0.0323	0.0155
Average	77.12	0.713	0.103	0.619	0.0990	0.0695	0.0295	0.0654	0.0494	0.0160
Low	70.53	0.483	0.026	0.402	0.0731	0.0430	0.0177	0.0397	0.0228	0.0108
High	82.93	0.979	0.205	0.997	0.144	0.0937	0.0503	0.0915	0.0768	0.0231

*All percentages are given on the basis of the fresh sample. The Mn figures are in parts per million and are given on a dry basis. **W = total content of aerial portion; E = calculated extractable material; R = results on residue from water extraction. We are indebted to Mr. Lester Nabors for the analyses on the residues.

TABLE I (continued)—Showing the Analyses of Wheat Plants*

Date of Sample	P %			Na %			K %			Mn ppm (Dry Basis)
	W	E	R	W	E	R	W	E	R	W
12-5-47	0.1160	0.1130	0.00303			0.0382	0.771	0.722	0.0492	51
1-2-48			0.00150			0.0561	0.983	0.920	0.0632	76
2-19-48	0.0961	0.0940	0.00208	0.268	0.229	0.0389			0.0463	50
3-1-48	0.0968	0.0951	0.00168	0.244	0.199	0.0447			0.0394	
3-8-48	0.0924	0.0906	0.00181	0.299	0.238	0.0611	0.758	0.716	0.0416	
3-15-48	0.0965	0.0944	0.00215	0.286	0.241	0.0453	0.840	0.791	0.0489	
3-22-48	0.0844	0.0826	0.00179			0.0460	0.919	0.869	0.0498	
3-29-48	0.0909	0.0895	0.00137	0.381	0.350	0.0308	1.077	1.035	0.0417	
4-5-48	0.0796	0.0780	0.00161	0.192	0.151	0.0409	0.997	0.958	0.0391	
4-12-48	0.0916	0.0899	0.00166	0.245	0.196	0.0489	1.143	1.088	0.0548	
10-7-48	0.0872	0.0856	0.00163			0.0379	1.686	1.660	0.0256	91
10-21-48	0.0917	0.0903	0.00143			0.0384	0.803	0.759	0.0438	70
11-4-48	0.0932	0.0917	0.00149	0.209	0.180	0.0290	0.827	0.787	0.0395	66
11-18-48	0.0888	0.0872	0.00161			0.0500	0.958	0.915	0.0429	81
12-9-48	0.1050	0.1034	0.00164	0.215	0.156	0.0595	0.818	0.770	0.0478	104
12-22-48	0.1060	0.1037	0.00234	0.196	0.139	0.0569	1.039	0.975	0.0640	110
2-28-49	0.1290	0.1269	0.00211	0.138	0.108	0.0303	0.775	0.720	0.0547	
3-14-49	0.1120	0.1105	0.00152	0.130	0.101	0.0289	0.864	0.818	0.0462	
3-28-49	0.1040	0.1024	0.00162	0.107	0.07230	0.0347	0.901	0.854	0.0470	74
4-11-49	0.0916	0.0902	0.00137			0.0348			0.0347	72
Average	0.0975	0.0957	0.00177	0.223	0.181	0.0426	0.950	0.903	0.0460	77
Low	0.0796	0.0780	0.00137	0.107	0.07230	0.0289	0.758	0.716	0.0256	50
High	0.1290	0.1269	0.00303	0.381	0.350	0.0611	1.686	1.660	0.0640	110

*All percentages are given on the basis of the fresh sample. The Mn figures are in parts per million and are given on a dry basis. **W = total content of aerial portion; E = calculated extractable material; R = results on residue from water extraction. We are indebted to Mr. Lester Nabors for the analyses on the residues.

cate that wheat plants supply a more nearly adequate portion of the sodium required by animals than do some of the other grasses.

SUMMARY

1) Analytic figures on some of the mineral components of wheat plants are given.

Figures are given for the mineral content of water-extracted residues from wheat plants, and the calculated values for the extractable minerals are listed.

2) Wheat is capable of supplying the quantitative requirements of cattle for calcium, magnesium, and phosphorus.

3) The ratio between water-soluble calcium and phosphorus does not favor the maximum absorption of these two elements.

4) The ratio of magnesium to calcium is not such that it favors the maximum absorption of calcium.

5) The potassium content of wheat is high in comparison with certain grasses, but the sodium content is also high, and the ratio of potassium to sodium is lower in wheat than in the grasses.

TABLE 2—A Comparison of the Mineral Requirements of a Beef Cow* and the Minerals Furnished by Wheat Plants

	Gm. Ca	Gm. P	Ca/P ratio	Gm. Mg	Mg/Ca ratio	Gm. K	Gm. Na	K/Na ratio
Minerals required by 1,600-lb. cow per day	20	24		6				
Total minerals supplied by 140 lb. of wheat**	63	62	1.02	42	0.66	603	142	4.26
Soluble minerals in 140 lb. of wheat	44	61	0.73	31	0.71	573	115	4.98

*Recommended Nutrient Allowances for Domestic Animals, No. IV, Recommended Nutrient Allowances for Beef Cattle, National Research Council, Washington, D. C.

**Woodward, T. E., J. Dairy. Sci., *19*, (1936):347-357, finds that dairy cattle will consume about 140 lb. of grass per day.

References

[1]McMillen, W. N., and Langham, Wright: Grazing Winter Wheat with Special Reference to the Mineral Blood Picture. J. Anim. Sci., *1*, (1942):14-21.

[2]Harbaugh, F. G., and Dennis, Joe: The Blood Potassium and Calcium Levels of Cattle Grazing Wheat. Am. J. Vet. Res., *8*, (1947):396-399.

[3]Sjollema, B.: On the Nature and Therapy of Grass Staggers. Vet. Rec., *10*, (1930):425-430.

[4]Dennis, Joe, and Harbaugh, F. G.: The Experimental Alteration of Blood Potassium and Calcium Levels in Cattle. Am. J. Vet. Res., *9*, (1948):20-25.

[5]Methods of Analysis. A. Official Agric. Chemists, 5th ed., (1940):353.

[6]Methods of Analysis. A. Official Agric. Chemists, 3rd ed., (1931):104; 4th ed., (1935):21, 124-126.

[7]Stuart, Margaret R.: An Investigation of the Mineral Composition of Wheat Plants. A thesis, Texas Technological College, Lubbock, Texas.

[8]Pearson, P. B., Gray, J. A., and Reiser, Raymond: The Calcium, Magnesium and Potassium Contents of the Serum of Ewes Fed High Levels of Potassium. J. Anim. Sci., *8*, (1949):52-55.

[9]Burkhart, H. R.: Personal communication.

[10]Fenn, W. O.: The Role of Potassium in Physiological Processes. Physiol. Rev., *20*, (1940):377-415.

[11]Haag, J. R., and Palmer, L. S.: The Effect of Variations in Proportions of Calcium, Magnesium, and Phosphorus Contained in the Diet. J. Biol. Chem., *76*, (1928):367-389.

[12]Palmer, L. S., Eckles, C. H., Schutte, D. J.: Magnesium Sulfate as a Factor in Retention of Calcium and Phosphorus in Cattle. Proc. Soc. Exptl. Biol. & Med., *26*, (1928):58-62.

[13]Riggs, T.: Sixth Annual Report of the Mineral Contents of Pastures Investigation at the Cawthron Institute. New Zealand Dept. Sci. Ind. Res., Eighth Ann. Rep., (1934):18-20.

[14]Thornock, Charlotte, Guerrant, N. B., and Butcher, R. Adams: The Effect of Manganese on Calcification in the Growing Rat. J. Nutr., *25*, (1942):445-458.

[15]Reid, J. T., Pfau, K. O., Salsbury, R. L., Bender, C. B., and Ward, G. M.: The Effect of Manganese and Other Trace Elements on the Metabolism of Calcium and Phosphorus During Early Lactation. J. Nutr., *34*, (1947):661-676.

[16]Morrison, F. B.: Feeds and Feeding. Morrison Publishing Co., New York, 20th ed., (1945):1027.

Studies of Canine Leptospirosis
I. Evaluation of Laboratory Diagnostic Procedures
II. Serologic Determination of the Incidence of Latent Infection in the Lansing, Michigan Area

JOHN P. NEWMAN, D.V.M., M.S.

East Lansing, Michigan

PRIOR TO JULY, 1945, canine leptospirosis had only been diagnosed clinically in the Lansing area. On July 3, 1945, a dog showing icterus was admitted to the veterinary clinic at Michigan State College and leptospiras were demonstrated by microscopic and cultural methods. The organism appeared to be *Leptospira canicola* upon serologic examination.

It was soon apparent that because of the similarity in clinical symptoms of canine leptospirosis, canine distemper, black tongue, and various gastrointestinal disturbances, an accurate differential diagnosis, based on clinical evidence alone, was often impossible. As a result, the immediate instigation of specific therapeutic measures was not possible.

Many laboratory procedures have been employed as aids in the diagnosis of leptospirosis. However, an evaluation of them was not available. Experiment 1 was planned to evaluate the relative merits of the various laboratory procedures as rapid, accurate, diagnostic aids in work with canine leptospirosis.

The possibility that many dogs in this area were excreting the organisms in their urine, thus serving as a source of infection for dogs and man, was considered important. Meyer *et al.*,[25] reported that serums obtained from 10 dogs in the Detroit area were serologically positive to *Leptospira icterohaemorrhagiae*. Since that time, no further studies in Michigan have been reported. Experiment 2 was planned to determine the incidence of latent canine leptospirosis in the Lansing area.

Published with the permission of the director of the Experiment Station as journal article No. 1044 (n.s.). Condensed from a thesis submitted to the School of Graduate Studies of Michigan State College in partial fulfillment of the requirements for the degree of master of science.

From the Section of Bacteriology, Michigan Agricultural Experiment Station, East Lansing.

Presented before the Section on Research, Eighty-sixth Annual Meeting, American Veterinary Medical Association, Detroit, Mich., July 11-14, 1949.

REVIEW OF LITERATURE

The Disease.—Twenty-eight years after Weil described the disease in man, which bears his name, Inada *et al.*,[16] Huebener and Reiter (cited by Walch-Sorgdrager),[13] and Uhlenhuth and Fromme[42] isolated the causative agent. Noguchi[29] isolated the same organism from wild rats in New York City and proposed the name, *Leptospira icterohaemorrhagiae*.

Krumbein and Frieling,[20] in 1916, Courmont and Durand,[5] Lukes,[22] and Krivacek[19] suspected that Weil's disease (leptospirosis) may occur in dogs. Okell, Dalling, and Pugh,[30] in 1925, proved that infectious jaundice, occurring naturally in dogs, was caused by a spirochete which corresponded in every way to *L. icterohaemorrhagiae* obtained from rats.

Klarenbeek and Schüffner,[17] in 1931, were the first to recognize and describe the symptoms and pathology of canine leptospirosis, as caused by a new species. This species is now known as *L. canicola*, the epidemiologic importance of which was emphasized by Klarenbeek,[18] who demonstrated that *L. canicola* may also produce infection in human beings.

Reports of the occurrence of canine leptospirosis in the United States have been comparatively recent. Jungherr[15] reported an outbreak in a Pointer kennel and demonstrated a spirochete in the tissues from the dogs which died of the disease. Elander and Perry[10] and Bloom[3] reported a study of 6 and 9 cases, respectively.

The incidence of latent canine leptospirosis in Pennsylvania was reported by Raven,[31] in southern California by Greene,[11] and in war dogs by Jones *et al.*[14]

Meyer *et al.*[25] were the first to isolate *L. canicola*, and Randall[32] the first to isolate *L. icterohaemorrhagiae* from dogs in this country.

Serologic Tests.—The adhesion, complement-fixation, and agglutination-lysis tests have been employed in the serologic identification of Leptospira organisms and diagnosis of infection. Brown and Davis[4] and Brown[4] employed the adhesion test in the serologic examination of spirochetes and the serologic diagnosis of Weil's disease in man, respectively. Noguchi,[29] Bessemans and Nelis,[2] and Pot and Dornick[31] successfully employed the comple-

ment-fixation test. Schüffner and Mochtar[20] gave a thorough account of the microscopic agglutination-lysis test employing live Leptospira antigen and Walch-Sorgdrager[21] employing formalin-killed Leptospira antigen. The macroscopic agglutination test was described and employed by Pot,[16] Smith and Tulloch,[17] Brown,[5] and Starbuck and Ward.[11] Starbuck and Ward compared the macroscopic plate and the standard microscopic agglutination tests and, though their results indicated that the macroscopic plate test was not as accurate as the microscopic test, it was sufficiently reliable to be useful in the diagnosis of leptospiral jaundice in human beings.

MATERIALS AND METHODS

Preparation of Blood and Urine.—Blood and urine on which the microscopic, cultural, and serologic examinations were conducted in experiment 1 were obtained from Leptospira-infected dogs. Eight to 10 ml. of blood and 20 to 30 ml. of urine were obtained aseptically and centrifuged at 1,600 r.p.m. for ten minutes to remove the blood cells and extraneous material, then at 2,800 r.p.m. for one hour to throw down the Leptospira organisms. The supernatant serum was saved for serologic studies and the supernatant urine was discarded. Of the remaining sediments, 0.01 ml. was placed on each of three glass slides, which were stained with Giemsa's stain, Fontana's silver stain, and examined by dark field illumination, respectively. Known positive and negative slides were employed as controls in conjunction with each group of stained slides.

Three to 5 ml. of blood were drawn aseptically from the apparently normal dogs examined in experiment 2 and allowed to clot. The serum was removed and examined within eight hours.

Isolation of the Organism.—One-milliliter amounts of the remaining blood and urine sediments were suspended in 2 ml. of Schüffner's medium (minus rabbit serum) and used to inoculate two tubes of Schüffner's modification of Verwoort's medium (cited by Meyer[22]). All tubes were incubated at 30 C. with periodic macroscopic and dark field examinations performed to determine the presence or absence of growth. Three transfers to new cultural medium were made at seven-day intervals and all culture tubes were held for four weeks before being considered as negative.

Preparation of Antigen.—A clear medium which will support heavy leptospiral growth, free from clumps and débris, is most desirable for antigen production. Schüffner's modification of Verwoort's medium was found superior in trials conducted with the mediums recommended by Noguchi and Battistini,[6] Korthof,[7] Starbuck and Ward,[11] and Chang.[3]

Fresh antigen prepared from four- to five-day-old cultures of *L. canicola* and *L. icterohaemorrhagiae* was employed in all serologic tests.* The organisms were killed by adding neutral formalin to a final concentration of 0.15 per cent and then were centrifuged for five to ten minutes at 1,600 r.p.m. to remove clumps of organisms and extraneous materials which might interfere with the interpretation of the test. All tubes in which the organism showed atypical morphology, poor growth, or in which bacterial contamination was observed were discarded.

Agglutination Test.—The agglutination test, as outlined by Schüffner and Mochtar,[20] employing formalin-killed antigen, was conducted on all serums examined. The serum-antigen dilutions were shaken for five minutes and then incubated in a 37 C. water bath for three hours. One loopful of each dilution was placed on a clean glass slide and covered with a No. 1, 22-mm. square cover glass and examined by dark field illumination.

The varying degrees of agglutination observed were arbitrarily given values, as suggested by Meyer,[22] to standardize the readings as follows:

One plus—small clumps of 3 to 10 Leptospira organisms and many free ones;

Two plus—numerous small clumps of leptospiras and many free ones;

Three plus—numerous large clumps of leptospiras and few free ones;

Four plus—numerous large clumps of leptospiras and only an occasional free one.

Positive and negative control serums were employed in conjection with each set of agglutination tests.

RESULTS OF EXPERIMENT 1

In an attempt to evaluate some of the laboratory diagnostic procedures, microscopic, cultural, and serologic examinations were conducted on the serum and microscopic and cultural examinations on the urine of 30 naturally infected dogs.

Of the 30 dogs examined, 9 were found to be infected with leptospiras, as demonstrated by one or more of the methods employed. Leptospira organisms could not be detected in any of the serum preparations using Giemsa's stain but they could be detected with Fontana's silver stain in 3 (10.0%) of the cases. Dark field examination revealed the presence of leptospiras in 7 (23.3%) serum preparations and in 1 (3.3%) urine preparation. In 1 case, the urine sediment was found to contain leptospiras by all three procedures.

*The original antigen cultures were obtained through the courtesy of Dr. K. F. Meyer of the George Williams Hooper Foundation.

The results of the cultural examinations of serum compare favorably with those obtained by dark field examination. Leptospiras were isolated from 6 (20.0%) of the dogs. All these cultures were *L. canicola*. They were identified serologically. Negative results were obtained in attempts to isolate the organisms from urine sediment, due to bacterial contamination.

Serum samples for agglutination tests were obtained at three intervals. The first sample was obtained when the dog was admitted to the clinic and the second and third samples were obtained one and two weeks thereafter. The results of the agglutination tests are summarized in table 1. The results of the first test designate the highest dilution in which agglutination was observed. In the second and third tests, only the highest dilution in which a four plus (++++) agglutination reaction occurred was recorded.

A four plus agglutination reaction in a 1 : 100 dilution, or higher, was obtained with 14 samples in the first test, 16 in the second test, and 30 in the third test. The agglutination titer observed in the three tests varied considerably, ranging from negative to four plus in 1 : 100, four plus in 1 : 10 to 10,000, and four plus in 1 : 100 to 30,000 serum dilutions, respectively. The titer increased as the disease progressed, with the maximum agglutination occurring in the higher dilutions in the third test.

The dogs ranged in age from 6 months to 7 years, and 16 (53.5%) were males. The dogs were arbitrarily divided into three age groups as follows: 1 month to 1 year, 9 (30%) dogs; 1 year through 3 years, 14 (46.7%) dogs; and 4 years through 7 years, 7 (23.3%) dogs.

The occurrence of specific and paraspecific reactions is summarized in table 2. These results are based on the reactions observed for each antigen in the third agglutination test. It was noted that 20 (66.7%) of the serums showed paraspecific reactions with *L. icterohaemorrhagiae*. Furthermore, as the specific titer increased, there was also an accompanying increase in titer and frequency of the paraspecific reactions.

RESULTS OF EXPERIMENT 2

In an attempt to ascertain the magnitude of reservoirs of leptospiral infection in the Lansing area, serologic studies were conducted on the serums obtained from 500 dogs. These dogs were hospitalized either in the veterinary clinic at Michigan State College or in nearby kennels. At the time of bleeding, all dogs appeared normal and none showed clinical evidence of leptospirosis. History relative to leptospirosis was unobtainable in most instances. Subsequent tests were not conducted on any of the dogs.

Five milliliters of blood were drawn aseptically from the radial vein of each dog and allowed to clot. The serum was removed

TABLE 1.—Serologic Results of Three Tests Made at Six- to Eight-Day Intervals on 30 Leptospira-Infected Dogs

No.	1st Test	2nd Test	3rd Test	Sex	Age
1	1+,	0	0	M	6 mos.
	0	4+, 1:10	4+, 1:300		
2	3+, 1:30	4+, 1:10	4+, 1:3,000	F	3 yrs.
	4+, 1:100	4+, 1:300	4+, 1:10,000		
3	3+, 1:10	4+, 1:300	4+, 1:300	M	4 yrs.
	4+, 1:300	4+, 1:10,000	4+, 1:30,000		
4	0	0	0	M	1 yr.
	0	4+, 1:30	4+, 1:300		
5	0	0	0	F	6 mos.
	0	4+, 1:30	4+, 1:300		
6	0	0	0	F	1 yr.
	0	4+, 1:10	4+, 1:1,000		
7	1+, 1:10	4+, 1:10	4+, 1:100	F	6 mos.
	0	4+, 1:30	4+, 1:300		
8	1+, 1:10	4+, 1:10	4+, 1:1,000	M	2 yrs.
	3+, 1:30	4+, 1:100	4+, 1:10,000		
9	1+, 1:10	4+, 1:300	4+, 1:1,000	F	2 yrs.
	4+, 1:30	4+, 1:300	4+, 1:10,000		
10	0	0	0	M	11 mos.
	0	4+, 1:30	4+, 1:100		
11	3+, 1:30	4+, 1:300	4+, 1:3,000	M	7 yrs.
	3+, 1:100	4+, 1:1,000	4+, 1:30,000		
12	4+, 1:30	4+, 1:100	4+, 1:100	M	3 yrs.
	4+, 1:30	4+, 1:100	4+, 1:1,000		
13	3+, 1:10	4+, 1:10	4+, 1:3,000	M	1 yr.
	3+, 1:30	4+, 1:30	4+, 1:10,000		
14	2+, 1:10	4+, 1:300	4+, 1:1,000	F	2 yrs.
	2+, 1:30	4+, 1:1,000	4+, 1:10,000		
15	0	0	0	M	5 yrs.
	0	4+, 1:100	4+, 1:1,000		
16	0	0	0	M	6 mos.
	0	4+, 1:30	4+, 1:300		
17	1+, 1:10	4+, 1:10	4+, 1:30	F	4 yrs.
	2+, 1:30	4+, 1:100	4+, 1:1,000		
18	0	0	0	M	1 yr.
	2+, 1:30	4+, 1:30	4+, 1:1,000		
19	0	3+, 1:10	4+, 1:1,000	F	2 yrs.
	0	4+, 1:30	4+, 1:10,000		
20	1+, 1:300	4+, 1:300	4+, 1:10,000	F	4 yrs.
	2+, 1:300	4+, 1:300	4+, 1:30,000		
21	0	0	0	F	11 mos.
	0	4+, 1:30	4+, 1:300		
22	0	4+, 1:300	4+, 1:3,000	M	2 yrs.
	0	4+, 1:300	4+, 1:10,000		
23	0	4+, 1:100	4+, 1:300	F	6 yrs.
	0	4+, 1:300	4+, 1:1,000		
24	0	4+, 1:30	4+, 1:100	F	10 mos.
	0	4+, 1:300	4+, 1:1,000		
25	0	0	0	F	7 mos.
	0	4+, 1:300	4+, 1:300		
26	3+, 1:10	4+, 1:30	4+, 1:1,000	M	1 yr.
	3+, 1:30	4+, 1:300	4+, 1:3,000		
27	1+, 1:10	4+, 1:30	4+, 1:300	F	5 yrs.
	0	4+, 1:30	4+, 1:1,000		
28	0	3+, 1:10	4+, 1:30	M	9 mos.
	0	4+, 1:30	4+, 1:1,000		
29	0	3+, 1:30	4+, 1:100	M	5 yrs.
	0	4+, 1:30	4+, 1:1,000		
30	0	4+, 1:100	4+, 1:300	M	1 yr.
	0	4+, 1:1,000	4+, 1:3,000		

*First line, titer of sero-reaction for *Leptospira icterohaemorrhagiae* antigen; second line for *Leptospira canicola*.

and refrigerated at 4 C. The agglutination test was conducted on all serum samples within three days after collection.

Formalin-killed *L. canicola* and *L. icterohaemorrhagiae* antigens were employed in the agglutination test. A one plus (+) or stronger reaction in the 1 : 10 or higher serum dilution was considered indicative of a latent infection.

221 (44.2%) female dogs tested, 44 (29.6%) gave a positive agglutination reaction with one or both antigens. Forty (90.0%) gave a positive reaction with *L. canicola* antigen, 3 (6.8%) with *L. icterohaemorrhagiae*, and 1 (2.3%) showed an equal titer with both antigens. The proportion of male to female reactors was slightly more than 2 : 1.

TABLE 2—Titer of Specific and Paraspecific Reactions in 30 Dogs (Active Infection with *Leptospira Canicola*)

Specific *L. canicola* titer	Total No. reacting	Paraspecific *L. icterohaemorrhagiae* titer							
		Neg.	10	20	100	200	1,000	2,000	10,000
100	4	4	0	0	0	0	0	0	0
200	4	3	0	0	1	0	0	0	0
1,000	6	2	0	1	2	1	0	0	0
2,000	6	1	0	0	1	2	2	0	0
10,000	8	0	0	1	0	1	3	3	0
20,000	2	0	0	0	0	0	0	1	1
Total	30	10	0	2	4	4	5	4	1

A majority of the positive serums showed a stronger reaction to one antigen than to the other. In these instances, the antigen which gave the stronger reaction in the highest dilution was considered to be specific. The antigen giving the weaker reaction with the same serum was considered to be paraspecific.

Of the 500 serums examined, 148 (29.8%) gave a positive reaction with one or both antigens. The remaining 352 (80.4%) gave no reaction with either antigen. One hundred thirty-one (26.2%) showed a stronger reaction with *L. canicola* antigen and 13 (2.6%) a stronger reaction with *L. icterohaemorrhagiae* antigen. Four of the samples (0.8%) reacted with an equal intensity with both the *L. canicola* and *L. icterohaemorrhagiae* antigens. The results are presented in table 3.

The dogs were arbitrarily placed in four age groups. The incidence of infection in the four groups was: 1 month to 1 year, 26.9 per cent; 1 year through 3 years, 17.4 per cent; 4 years through 7 years, 42.6 per cent; and 8 years through 12 years, 57.4 per cent. These data indicate that the older the dog, the greater the opportunity for exposure. Of the 279 (53.8%) male dogs tested, 104 (70.4%) gave a positive serologic test with one or both antigens. Ninety-one (89.5%) of the male reactors were positive to *L. canicola* antigen, 10 (9.6%) to *L. icterohaemorrhagiae*, and 3 (2.88%) showed an equal titer with both antigens. Of the

Paraspecific reactions, especially in the higher dilutions, occurred with serums giving a specific titer with both antigens. Of the samples, 75 (57.3%) gave positive paraspecific reactions to *L. canicola* and 5 (38.4%) of the samples were positive to *L. icterohaemorrhagiae*. The *L. canicola* positive serums showed the highest percentage of paraspecific reactions. No matter which antigen was used, paraspecific reactions occurred more commonly in serums showing high specific titers.

TABLE 3—Specific and Paraspecific Reactions of 148 Serologically Positive Dog Serums Samples

Specific positive titers	No. of samples	Per cent of reactors	Per cent of total
L. icterohaemorrhagiae	13	8.8	2.6
L. canicola	131	88.5	26.2
L. icterohaemorrhagiae and *L. canicola* equal	4	2.7	0.8
Total	148		29.6

DISCUSSION

The results of experiment 1 indicate that none of the laboratory diagnostic procedures are reliable at all times. Only 10 per cent of the stained blood sediment and 2.3 per cent of the dark field blood sediment preparations showed Leptospira organisms. False positives in stained smears are practically nonexistent, although caution should be exercised in the interpretation of dark field preparations. Schultz[39] and Hall[12] observed

motile filamentous elements, "pseudospirochetes," in the blood of normal and diseased individuals. If due cognizance is taken of the possible presence of these pseudospirochetes, fewer false positives will be recorded.

Cultural examinations of blood yielded Leptospira organisms in 6 (20.0%) of the dogs examined. If the blood could have been obtained on or before the fourth or fifth day of illness, it is possible that a greater percentage of isolations would have been obtained. This confirms the results of other workers in their attempts to isolate Leptospira organisms from human beings (Davidson *et al.*,[9] Smith and Tulloch,[40] Packchanian,[33] and Bertucci[1]).

Table 1 indicates that the accuracy of the agglutination test increases as the disease progresses. The results show that at twelve to fifteen days and twenty-one to thirty days of illness, respectively, the test was approximately 50 and 100 per cent accurate. According to Bertucci, most workers agree that strong false positive reactions are nonexistent. If agglutination tests are conducted with serum collected at different intervals, a more accurate determination of the status of the infection may be made.

Although the agglutination test is a rapid, accurate, laboratory diagnostic procedure, it must be emphasized that its usefulness is limited. At the time when a positive diagnosis can be made by this test, most of the animals have either died or are on their way to recovery. Hence, the immediate instigation of specific therapeutic measures is not possible. However, as the results of experiment 1 and 2 indicate, it would appear that it is fully reliable in retrospective diagnoses.

The results of experiment 2, relative to the sex incidence of the disease, are in accord with the findings of Meyer[24] and Raven.[37] Their examinations also dealt predominantly with *L. canicola* infections. Klarenbeek[16] reports that leptospirosis may occur up to ten times more often in the male than in the female. However, *L. icterohaemorrhagiae* infections predominated in the animals studied by him.

It has been reported by Meyer *et al.*,[24] Raven,[37] and Walch-Sorgdrager[43] that infections due to *L. canicola* yield a higher titer than those due to *L. icterohaemorrhagiae*. The results of the third agglutination test in experiment 1 showed that 22 (73.3%) of the serums gave four plus reactions in dilutions of 1 : 1,000, or higher. In experiment 2, only 2 (15.4%) of the serums had a 1 : 10,000, or greater, specific titer to *L. icterohaemorrhagiae*. Only 35 (26.7%) of the serums had a 1 : 1,000 or greater specific titer with *L. canicola*.

Attention is called to the 1-year through 3-year age group of 221 (44.7%) dogs, reported in experiment 2, which does not conform to the expected pattern of age incidence. Seventeen per cent of this group showed Leptospira antibodies. Attention is also directed to the fact that 25.7 per cent of the positive agglutination reactions occurred in this group. The reason for this could possibly be explained by the fact that kennel dogs were included in this experiment. The ages of the majority of these dogs ranged from 1 year through 3 years. These dogs were kept in kennels and runs with concrete floors and rat infestation was not evident. Since they were not allowed to run free and roam, as many pet dogs are, perhaps they had less chance of exposure

TABLE 4—Incidence of Latent Canine Leptospirosis

State or country	Positive *L. canicola* (No.)	(%)	Positive *L. icterohaemorrhagiae* (No.)	(%)	No. dogs examined
Holland[a]	25	32.0	10	13.0	76
Holland[b]	10	20.0	4	8.0	50
Germany[b]	6	12.0	3	6.9	48
Germany[b]	7	3.5	1	0.5	200
Germany[b]	1	1.9	18	34.0	53
Denmark[b]	0	0.0	0	0.0	21
Alabama[b]	20	26.0	0	0.0	75
California[b]	10	9.0	3	2.7	111
New York[b]	11	22.0	3	6.0	50
Pennsylvania[b]	26	24.8	9	8.5	105*
Pennsylvania[b]	0	0.0	10	76.0	13
Michigan[b]	131	26.8	13	2.6	500**
Michigan[b]					

[a]Dogs from the urban area, reported by Raven.[37] [b]Dogs from the rural area, reported by Raven.[37] [c]Reported by Meyer.[24] [d]Reported by author. *Five serums demonstrated an equal titer for both antigens. **Four serums demonstrated an equal titer for both antigens.

to the infection. It is possible that, if kennel dogs had not been included in this study, the number of positive dogs would have been greater in this age group.

The incidence of latent canine leptospirosis has been summarized from comparable reports in table 4. The world incidence is 0 to 45 per cent, the highest incidence occurring in Holland, where perhaps the most extensive studies on both human and canine leptospirosis have been conducted. In the United States, the range is 0 to 33.3 per cent, with the highest reported from Pennsylvania and none reported from Alabama. The results reported here correlate closely with other reports from this country, although the incidence of *L. icterohaemorrhagiae* canine infection is slightly lower.

There appears to be an explanation for the difference between the results obtained by Meyer[24] and the results reported here. The ten samples examined by Meyer were obtained from the Detroit area, from which Molner and Kasper,[26] Molner and Meyer,[27] and Molner, Meyer, and Raskin[28] reported 75 human cases of Weil's disease. In addition, Meyer *et al.*,[24] reported that 16 per cent of the rats in that area showed renal leptospiral infections. Thus, because of environmental conditions which exist in the Detroit area, the incidence of *L. icterohaemorrhagiae* infections is higher than in the Lansing area. It should be mentioned, however, that the canine serum samples submitted to the author for examination from the Detroit area indicate that *L. canicola* infections are also present.

Evidence supported by the findings in experiment 2 (table 3) indicates that many of the cases of typical canine illness may be subclinical or latent leptospirosis. Leptospiruria occurs in these dogs and they play a predominant role as spreaders of the disease to other dogs and to human beings.

SUMMARY

Staining, cultural, and serologic procedures were conducted on the blood and urine collected from 30 naturally infected dogs to determine the value of these procedures for the laboratory diagnosis of canine leptospirosis.

The staining and cultural procedures are not considered reliable diagnostic methods. The agglutination test was approximately 50 per cent accurate after the second week of illness and 100 per cent accurate after the third to fourth week.

The serums from 500 normal dogs were examined by the agglutination test in retrospective diagnosis. Of the 29.6 per cent of the dogs demonstrating some titer to either *Leptospira canicola* or *Leptospira icterohaemorrhagiae* antigens, or both, 26.2 per cent were positive with *L. canicola*, 2.6 per cent were positive with *L. icterohaemorrhagiae*, and 0.8 per cent showed an equal titer with both antigens.

Male and female incidence of latent infection was approximately 2 : 1.

The incidence of the infection, as evidenced by the results of experiment 2, increased with the age of the dogs.

The results of experiment 2 indicate that nearly one third of the dogs in the Lansing area are possible reservoirs of Leptospira infection, from which other dogs and man may acquire leptospirosis.

References

[1]Bertucci, E. A.: Leptospirosis. Am. J. M. Sc., 209, (1945): 86-111.

[2]Bessemans, A., and Nelis, P.: Sur la fixation du complement dans *La Spirochetose icterohemorrhagique*. Compt. rend. Soc. de Biol., 98, (1928):1234-1236.

[3]Bloom, F.: The Histopathology of Canine Leptospirosis. Cornell Vet., 31, (1941): 268-288.

[4]Brown, H. C.: Serological Diagnosis of Weil's Disease. Adhesion Test Procedure. Brit. M. J., 1, (1935): 411-413.

[5]Brown, H. C.: A Rapid Presumptive Serological Test for Weil's Disease. Brit. M. J., 2, (1939): 1183-1184.

[6]Brown, H. C., and Davis, L. J.: The Adhesion Phenomenon as an Aid to the Differentiation of Leptospira. Brit. J. Exper. Path., 8, (1927):397-402.

[7]Chang, S. L.: Studies on *Leptospira Icterohaemorrhagiae*. I. Two New Mediums for Growing *Leptospira Icterohaemorrhagiae*, *Leptospira Canicola*, and *Leptospira Biflexor*, and a Method for Maintaining the Virulence of *Leptospira Icterohaemorrhagiae* in Culture. J. Infect. Dis., 81, (1947): 28-34.

[8]Courmont, J., and Durand, P.: *La Spirochetose Icterohemorragique* chez Le Chien. Compt. rend. Soc. de Biol., 82, (1917): 275-277.

[9]Davidson, L. S. P., Campbell, R. M., Rae, H. J., and Smith, J.: Weil's Disease (Leptospirosis), a Clinical and Bacteriological Study of 19 Cases Occurring Chiefly Among Fish Workers. Brit. M. J., 2, (1934): 1137-1142.

[10]Elander, R. J., and Perry, J. K.: Leptospirosis in Dogs. J.A.V.M.A., 96, (1940):311-314.

[11]Greene, M. R.: A Survey of Leptospirosis in Southern California. Am. J. Hyg., 34, (1941):87-90.

[12]Hall, M. W.: Spirochete-like Filaments in Blood of Dengue Patients and Normal Individuals. Am. J. Trop. Med., 5, (1925): 207. (Original not seen. Abstr. J.A.M.A., 85, (1925):1925.)

[13]Jones, T. F., Roby, T. O., Davis, C. L., and Maurer, F. D.: Control of Leptospirosis in War Dogs. Am. J. Vet. Res., 6, (1945):120-128.

[14]Inada, R., Ido, Y., Hoki, R., Kaneko, R., and Ito, H.: The Etiology, Mode of Infection, and Specific Therapy of Weil's Disease (Spirochaetosis Ictero-haemorrhagica). J. Exptl. Med., 23, (1916): 377-402.

[15]Jungherr, E.: Observations on Canine Spirochetosis in Connecticut. J.A.V.M.A., 44, (1937):661-673.

[16]Klarenbeek, A.: *Leptospirosis Icterohaemorrhagiae* (Weilsche Krankheit) beim Hunde. Proc. Twelfth Vet. Cong., 3, (1934):249-257.

[27]Klarenbeek, A., and Schuffner, W. A. P.: Het Voorkomen van een afwijkend Leptospirenras en Nederland, Nederl. tijdschr. v. geneesk., *3*, (1933): 4571-4572. (Original not seen. Klarenbeek, A.: Klinische Statistik der Leptospirosen des Hundes. Zentralbl. f. Bakt., *142*, (1938): 82-86.)

[28]Korthoff, G.: Experimentelles Schlammfieber beim Menschen. Zentralbl. f. Bakt., *125*, (1932): 429-434.

[29]Krivacek, O.: Spirochaeten befunde beim Hundetyphus. Ztschr. f. Hyg. u. Infectionskr., *102*, (1924): 529-532.

[30]Krumbein, R., and Frielling, B.: Zur Weilschen Krankheit. Deutsche med. Wchnschr., *19*, (1916): 564. (cited by Okell, C. C.: Leptospiral Jaundice in Dogs. Brit. Med. J., *1*, (1925): 266.)

[31]Lukes, J.: Sur la presence de spirochetes chez les chiens atteints de gastroenterite et sur le role pathogene possible de ces microorganisms. Ann. Inst. Pasteur, *38*, (1924): 523-528.

[32]Meyer, K. F.: Personal communication.

[33]Meyer, K. F., Eddie, B., and Stewart-Anderson, B.: Canine, Murine, and Human Leptospirosis in California. Proc. Soc. Exptl. Biol. and Med., *38*, (1938): 17-19.

[34]Meyer, K. F., Stewart-Anderson, B., and Eddie, B.: Epidemiology of Leptospirosis. Am. J. Pub. Health, *29*, (1939): 347-353.

[35]Meyer, K. F., Stewart-Anderson, B., and Eddie, B.: Canine Leptospirosis in the United States. J.A.V.M.A., *95*, (1939): 710-729.

[36]Molner, J. G., and Kasper, J. P.: An Outbreak of Jaundice in Detroit (A Preliminary Report). J.A.M.A., *110*, (1938): 2069-2070.

[37]Molner, J. G., and Meyer, K. F.: Jaundice in Detroit, Am. J. Pub. Health, *30*, (1940): 509-515.

[38]Molner, J. G., Meyer, K. F., and Raskin, H. A.: Leptospiral Infections, J.A.M.A., *136*, (1948): 814-819.

[39]Noguchi, H.: *Spirochaeta Icterohaemorrhagiae* in American Wild Rats and Its Relation to the Japanese and European Strain. J. Exptl. Med., *25*, (1917): 755-763.

[40]Noguchi, H.: Etiology of Yellow Fever. X. Comparative Immunological Studies on *Leptospira Icteroides* and *Leptospira Icterohaemorrhagiae*. J. Exptl. Med., *31*, (1920): 135-158.

[41]Noguchi, H., and Battistini, T. S.: Etiology of Oroya Fever. I. Cultivation of *Bartonella Bacilliformis*. J. Exptl. Med., *43*, (1926): 851-864.

[42]Okell, C. C., and Dalling, T.: Leptospiral Jaundice in Dogs (Yellows). Vet. J., *81*, (1925): 3-35.

[43]Packchanian, A.: Positive Agglutination Tests in Suspected Cases of Weil's Disease. Pub. Health Rep., *56*, (1941): 2145-2156.

[44]Pot, A. W.: A Macroscopic Agglutination Test in Weil's Disease. Lancet, *230*, (1936): 1280.

[45]Pot, A. W., and Dornick, G. J.: The Complement Fixation Test in the Diagnosis of Weil's Disease. J. Path. and Bact., *43*, (1936): 367-372.

[46]Randall, R., and Cooper, H. K.: The Golden Hamster as a Test Animal for Diagnosis of Leptospirosis. Science, *100*, (1944): 133-134.

[47]Raven, C.: Canine Leptospirosis in Pennsylvania. J. Infect. Dis., *69*, (1941): 131-137.

[48]Schüffner, W. A. P., and Mochtar, A.: Versuche zur aufteilung von Leptospirenstammen, mit einleitenden bemerkungen über den verlauf von agglutination und lysis. Zentralbl. f. Bakt., *107*, (1927): 405-413.

[49]Shultz, E. W.: The Pseudospirochetes Derived from Red Blood Cells. J. Lab. and Clin. Med., *8*, (1923): 375-381.

[50]Smith, J., and Tulloch, W. J.: A Macroscopic Agglutination Test for Diagnosis of Weil's Disease. Lancet, *211*, (1927): 846-850.

[51]Starbuck, E. B., and Ward, T. B.: A Comparison of a Recently Developed Macroscopic Agglutination Test for the Diagnosis of Leptospiral Jaundice with the Standard Microscopic Test. J. Infec. Dis., *70*, (1942): 88-91.

[52]Uhlenhuth, P., and Fromme, W.: Experimentelle untersuchungen über den infektionsmodus, die Epidemiologie und Serum behandlung der Weilschen Krankeit. Ztschr. f. Immunitätsforsch u. exper. Therap., *28*, (1919): 1-118.

[53]Walch-Sorgdrager, B.: Leptospirosis. Bull. Health Organ., *8*, (1939): 143-283.

A Study of Food Intake, Activity, and Digestive Efficiency in Different Type Dogs

W. T. JAMES, B.S., Ph.D., and C. M. McCAY, M.A., Ph.D.

Ithaca, New York

DOGS ARE excellent animals for many experimental purposes because there are so many different bodily types among them. Among dogs are found midget types, as the Pekingese; giant types, as the Great Dane and St. Bernard; and those which fall in what may be considered medium types, as the Basset Hound and the Terriers. They not only differ in size, but also in general bodily shape, and thus are valuable as experimental subjects for investigating the relationship between constitution and behavior. Consider the long, lean, thin-legged Saluki and Whippet in contrast to the heavy-set, short, stocky Basset Hound. These animals differ in form, and they also differ widely in psychology, as experiments carried on at Cornell University during 1932 to 1942 have shown.[1] During these experiments, much attention was given to the dietary requirements of the various types. In one of these experiments, which is reported here, an analysis was made of food intake, digestive efficiency, and general activity in three different breeds.

EXPERIMENTAL

The first experiment compared the food intake of the dogs when they were fed all they would eat for a number of days. Fifteen animals were used in the experiments, including 4 German Shepherds, 4 Salukis, 6 Basset Hounds, and 1 hybrid which was three-fourths Basset Hound and one-fourth German Shepherd, but which had the bodily shape of the Basset Hound. These dogs were given all the food they would eat each day for a period of five weeks. The results indicate that the dogs differ in food intake from 1 lb. of food for every 20 lb. of body weight, to 1 lb. of food for every 50 lb. of body weight. The breeds did not necessarily fall into the same grouping. For example, between food intake of 20 and 30 lb. of body weight to 1 lb. of food, there were 2 German Shepherds, 2 Salukis, and 2 young Basset Hounds. Between 30 and 50 lb. of body weight for every 1 lb. of food, there were 1 German Shepherd, 3 Salukis, 3 Basset Hounds, and 1 German Shepherd-Basset Hound hybrid.

The second problem was to determine whether these dogs could be maintained on an amount of food which had been found satisfactory in previous experiments on other breeds of dogs by C. M. McCay.[2] In this part of the experiment, the dogs were fed 1 lb. of food for 36 lb. of body weight for a period of twenty weeks. The weight of the dogs did not change appreciably during this period. It may be assumed therefore, in regard to weight, that many of the dogs were taking in more food in experiment 1 than they needed for maintenance. The dogs were apparently in as good health at the end of experiment 2 as in experiment 1.

In experiment 1, activity measurements were made on the dogs for a number of days. This was done by placing a pedometer in a small wooden locket which was suspended from the dog's neck by a strap. All of the pedometers had been graduated mechanically, and adjusted so they were equal in performance. Correlations were made on activity and food intake by the rank-difference method, with a coefficient of correlation of 0.72. This suggests the dogs which consumed the most food were in fact the most active of the group. This observation was substantiated in animals 2585 and 2586, 2 excitable German Shepherds which were trained in the conditioned reflex laboratory. These dogs were so excitable they could hardly be restrained in the Pavlov stand, in which other less active animals have been trained repeatedly. These animals were in a continuous state of movement in the kennel and difficult to restrain under laboratory conditions and they ranked first in activity.

The correlation between age and activity was 0.37, which suggests that, within the age limits involved, this was not the most important factor for the difference. In fact, the animal which ranked second in the group in activity (animal 1643, a German Shepherd) was as old as some of the Basset Hounds (1428 and 1426) which ranked on the bottom of the group in activity.

The correlation between weight and the pounds of food intake per pounds of body weight in experiment 1 was only 0.28.

The work, as a background for the writing of this paper, was done at the Cornell Behavior Farm, Cornell University, Ithaca, N. Y.

At present Dr. James is at the Department of Psychology, University of Georgia, Athens. Dr. McCay is in the Department of Animal Husbandry, Cornell University, Ithaca, N. Y.

The correlation between activity and weight was 0.64. In general, one might be inclined to assume the light animals would be more active than the heavier dogs. This does not seem to be the case, however, since some of the most active members of the group were also the heaviest.

All of these correlations would seem to indicate that it is rather difficult at the present time to make principles regarding food intake, activity, and weight. There are many factors involved in each of these, and it is extremely difficult to isolate them in one experiment. One of the most important differences between the dogs was that of general activity, and this does seem to be more closely related to food intake than any other factor.

The third experiment of the series was concerned with a comparison of the digestive efficiency of the dogs. In this experiment, the amount of food digested was determined on a typical Basset Hound, a German Shepherd, and a Saluki. The dogs were fed five different foods for a period of ten days and on the basis of 1 lb. of food for every 36 lb. of body weight. On the last six days of the period, the feces were collected, dehydrated, weighed, and then analyzed for their protein, starch, moisture, fiber, and other soluble extract, by the nutrition department of Cornell University. By comparing the amount of feces with the food consumed during the six-day period, the degree of digestion for each was determined for the dogs. The amount of food digested by each animal was relatively the same for all dogs. When fed on the basis of 1 lb. of food for 36 lb. of body weight, all dogs digested about 80 per cent of the food eaten. Table 1 shows the percentage of protein, starch, fiber, and fat content digested in each diet.

SUMMARY

1) The present study suggests that many dogs eat much more food than they need for maintenance if fed on the basis of self-selection.

2) The three breeds, Saluki, Basset Hound, and German Shepherd, can be maintained on 1 lb. of food for every 36 lb. of body weight without any difficulty.

3) There is not any great degree of difference between the breeds in the percentage of total food digested, or in the percentage of fat, protein, starch, and fiber content digested.

4) Apparently, the greatest difference between the dogs is general activity, which is rather highly correlated with food intake. This points to a difference in excitability as the important factor to deal with in studying the breeds.

TABLE I—Comparison of the Digestive Efficiency of the Dogs

Dog	Series*	Food eaten 6-day test (Gm.)	Feces dry wt. (Gm.)	Starch In food (Gm.)	Starch % dig.	Protein In food (Gm.)	Protein % dig.	Fiber In food (Gm.)	Fiber % dig.	Fat In food (Gm.)	Fat % dig.
Saluki	1	2718	587	924	98	693	72	104	35	108	83
	2	4344	748	1476	98	1107	78	166	45	173	86
	3	4062	868	1381	98	1035	73	156	37	162	80
	4	1878	345	455	97	531	78	130	58	160	87
	5	4062	901	1084	98	1113	70	108	##	154	73
German Shepherd	1	2718	559	924	98	693	75	104	36	108	79
	2	3971	583	1350	98	1012	82	146	60	158	95
	3	4734	921	1609	98	1207	76	182	44	199	83
	4	4746	1161	1150	96	1343	67	228	32	251	83
	5	4746	1114	1267	98	1301	72	127	##	180	79
Basset Hound	1	1868	386	535	98	476	75	71	38	74	84
	2	2886	649	981	98	736	70	101	33	115	81
	3	2888	628	981	98	735	72	111	38	115	85
	4	2880	698	698	97	815	69	138	28	152	70
	5	2954	552	789	97	810	78	78	28	112	84

Results were modified here because the animals began to take in hair from the body with the food. The reason for this is not known at present.

*In series 1, 2, and 3, the regular commercial food made by the Farm Products, Inc., was used. The food contained the following ingredients (percentage of each given): corn flakes, 33.2; wheat flakes, 23.0; meat meal, 10; liver meal, 10; soybean-oil meal, 5; dry skimmilk, 4; wheat germ, 4; tomato pomace, 2.5; dry yeast, 2; white fish meal, 2; cheese meal, 2; alfalfa meal, 0.5; bone meal, 1.0; salt, 0.4; and cod liver oil, 0.4. In series 4, 15 per cent soybean was added at the expense of corn and wheat flakes. In series 5, 12 per cent whey was added in place of corn and wheat flakes.

References

[1]James, W. T.: Morphological Form and Its Relation to Behavior. Am. Anatomical Mem. No. 19, (1941):525-643.

[2]McCay, C. M.: Nutrition of the Dog. Comstock Publishing Co., Ithaca, N. Y., 1949.

The Prophylactic and Therapeutic Properties of Sulfamerazine in Fowl Cholera

J. O. ALBERTS, V.M.D., Ph.D.

Urbana, Illinois

IN 1938, LEVADITI and Reinie[1] reported that sulfanilamide reduced losses from experimentally induced *Pasteurella multocida* infections of chickens. In the same year Cardosa, Nobrega, and Reis[2] reported similar results with sulfanilamide in an outbreak of fowl cholera in chickens. Unfortunately, large daily doses of sulfanilamide were needed, and such doses may cause paralysis or renal failure. Devolt[3] reported that 1 per cent sulfathiazole in the mash helped to control fowl cholera in chickens and turkeys, but 2 per cent was toxic to laying hens in three to four days.

It was felt that these findings justified the study of other sulfonamides. Sulfamerazine was selected, since it was readily available when the study was begun.

After this work was begun, Kiser *et al.*[4] reported that sulfamethazine was effective in reducing losses of chickens exposed to *P. multocida*. It was not toxic, and the disease did not recur. Later, Hilbert and Kiser[5] found sulfamethazine of value in a natural outbreak of fowl cholera in ducks.

EXPERIMENTAL METHODS

The chickens used in this study were of mixed breeds and were more than 7 months old. The turkeys were more than 6 months old, and were primarily of the Beltsville White variety, although some of the Broad Breasted Bronze variety were included.

The birds were confined indoors in unheated isolation quarters. They were fed a ration recommended by the University of Illinois, College of Agriculture, Division of Poultry Husbandry. Water was supplied *ad libitum*.

Strain 6-7 of *P. multocida*, which was used in this study, was isolated in 1945 from turkeys that had died during a field outbreak of acute fowl cholera. When grown on proteose-peptone agar containing 2 per cent horse serum, it produced colonies of the fluorescent type described by Hughes.[6] Lyophilized cultures were prepared by a slight modification of the method of Raper and Alexander.[7]

Adult chickens and turkeys were exposed to strain 6-7 of *P. multocida* either by intramuscular inoculation or by nasal instillation. This strain consistently killed turkeys within forty-eight hours after intramuscular inoculation of 5×10^6 or more viable organisms. Among similarly exposed chickens, no more than 60 per cent died within five days; however, as many as 30 per cent of the birds lingered as long as a month and developed symptoms of emaciation and either paralysis, encephalitis, or arthritis. Thirty-five per cent of the chickens inoculated intranasally died within seven days after exposure. An additional 25 to 30 per cent had rhinitis, ocular and orbital swellings, and arthritis, during the next three weeks.

Chickens and turkeys were challenged intramuscularly with approximately 5×10^6 to 2.5×10^7 live organisms, and intranasally with approximately 5×10^7 organisms. Necropsies and bacteriologic examinations of the liver, heart, lung, and blood were made of birds that died or were killed following exposure.

Sulfamerazine blood levels were determined according to the method of Bratton and Marshall.[8] Readings were made with a Klett-Summerson colorimeter.

EXPERIMENTAL RESULTS

Experiment A—Prophylactic Action of Sulfamerazine Against Experimental Fowl Cholera in Turkeys and Chickens.—Fewer losses were observed in turkeys simultaneously exposed to *P. multocida* by intramuscular injection and given sodium sulfamerazine orally than in untreated exposed turkeys. Birds that received 143.0 and 107.25 mg. of sodium sulfamerazine per kilogram of body weight (1.0 and 0.75 gr./lb.) in divided daily doses were better pro-

---

A section from a thesis submitted in partial fulfillment of the requirements for the degree of doctor of philosophy in the Graduate College of the University of Illinois.

From the Department of Veterinary Pathology and Hygiene, College of Veterinary Medicine and Agricultural Experiment Station, University of Illinois, Urbana.

Grateful acknowledgment is made of the advice and assistance given by Dean Robert Graham and Professor Norman D. Levine of the College of Veterinary Medicine, University of Illinois, and Dr. S. F. Scheidy, Glenolden, Pa.

Acknowledgment is made to Sharp and Dohme, Glenolden, Pa., for supplying the sulfamerazine used in this study and for supplemental financial aid by a research grant.

tected at the end of five and thirty days following exposure than those that received lower doses of 71.5 and 35.75 mg. per kilogram of body weight (table 1). Fowl cholera reappeared in the treated groups of turkeys within two to four days after therapy was stopped; however, losses subsided in groups that received an additional course of either 143.0 or 107.25 mg. of the drug per kilogram of body weight.

Three groups of 20, 20, and 10 yearling chickens were fed 0.4, 0.3, and 0.2 per cent sulfamerazine-mash mixtures, respectively, for two days before and five days after intramuscular exposure to *P. multocida* (table 2). Five days after exposure, the mortality was reduced 72.7, 54.5, and 27.2 per cent, respectively, in the three treated groups. Thirty days after exposure, the reductions in mortality were 50.0, 43.7, and 12.5 per cent, respectively.

In two similar groups of 20 chickens each, that received 0.2 or 0.1 per cent sodium sulfamerazine in the drinking water, mortality was reduced 72.7 and 27.2 per cent on the fifth day of treatment and 62.5 and 37.5 per cent, respectively, thirty days after exposure.

In table 3 are given the effects of sulfamerazine treatment in two groups of 20 chickens, each exposed intranasally. Five days after exposure, losses were reduced 83.3 and 100 per cent, respectively, in the birds that received 0.4 per cent sulfamerazine in the mash and 0.2 per cent sodium sulfamerazine in the drinking water. Thirty days after exposure or twenty-five days after treatment was stopped, losses were reduced 76.9 and 69.2 per cent, respectively, in the treated groups. At the end of thirty days, the survivors were killed. Blood cultures were negative for *P. multocida*. The organism was isolated from a lung abscess of 1 bird that was fed 0.4 per cent sulfamerazine in the mash and from the nasal cleft of 2 untreated, infected control birds.

Experiment B—Blood-Sulfamerazine Levels in Turkeys and Chickens.—Sulfamerazine concentrations in the blood were determined for four days in 5 turkeys of each group that received 143.0, 107.25, 71.5, and 35.75 mg. per kilogram of body weight (143.0 mg./kg. equals 1 gr./lb.) sodium sulfamerazine orally for two days (fig. 1). The drug was given in two divided doses at 8 a. m. and 6 p. m. and blood was collected two hours after the second dose was given. Although it was administered at a uniform dosage rate, differences were noted in the blood-sulfamerazine levels of different birds.

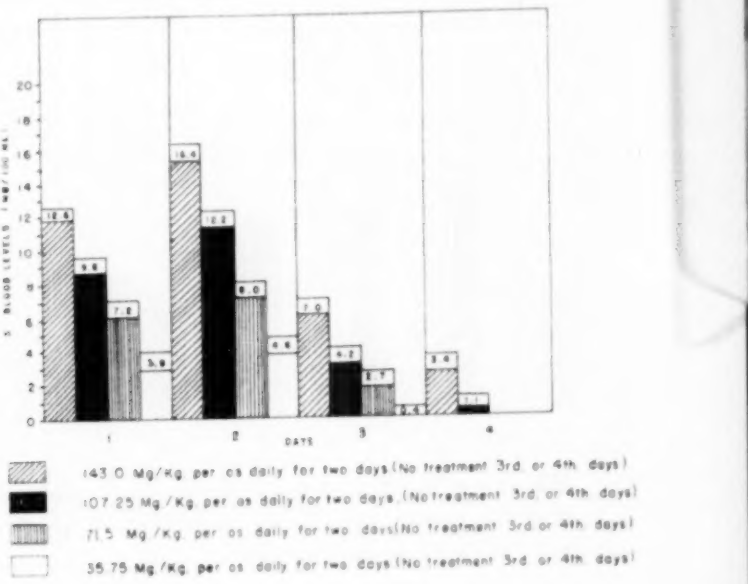


Fig. 1—Blood-sulfamerazine concentrations in turkeys.

Two hours after the fourth treatment, the highest mean blood-sulfamerazine level of 16.4 mg. per 100 ml. of blood was present in turkeys that had received sodium sulfamerazine at the rate of 143.0 mg. per kilogram of body weight. The average blood-sulfamerazine level was less than 5 mg. per 100 ml. of blood in turkeys that received 35.75 mg. per kilogram of body weight daily for two days. Two days after treatment was stopped, no sulfonamide was found in the blood of turkeys that had received 35.75 or 71.5 mg. of sodium sulfamerazine per kilogram of body weight.

Blood-sulfonamide levels were similarly determined in 5 chickens of each group that received 0.4 or 0.3 per cent sulfamerazine in the mash or 0.2 per cent sodium sulfamerazine in the drinking water (fig. 2). Differences in blood levels were noted among different chickens that had access to the same sulfamerazine-mash or water mixtures.

Experiment C—Therapeutic Action of Sulfamerazine and Sodium Sulfamerazine in Experimentally Induced Fowl Cholera of Chickens.—In two groups of 20 chickens each, exposed intramuscularly to approximately 1.5×10^8 *P. multocida*, therapy was delayed until symptoms of the disease appeared and a few birds had died. The therapeutic action of 0.4 per cent sulfamerazine in the mash or 0.2 per cent sodium sulfamerazine in the drinking water are shown in table 4. During the five-day period of access to sulfamerazine, 6 chickens died; however, 4 of the birds were visibly sick at the time treatment was begun. Similarly, 2 of 3 chickens which appeared sick at the time sodium sulfamerazine treatment was begun died during the following eighteen hours. Thirty days after exposure, losses were 21.3 per cent less in the chickens that had access to a 0.4 per cent sulfamerazine-mash mixture than they were in the untreated control birds. During the same period, losses were reduced 49.3 per cent in the birds that had received 0.2 per cent sulfamerazine in the drinking water.

Experiment D—Reëxposures of Turkeys Recovered from Experimental Pasteurella Multocida Infection.—Twenty-five turkeys that had recovered from an intramuscular injection of strain 6-7 with simultaneous sodium sulfamerazine therapy administered at the rate of 143.0 mg. per kilogram of body weight (*see* table 1) were reëxposed to the organism five weeks later. The birds were separated into five groups of 5 birds each and challenged by intramuscular injection of 2.5×10^8, 8×10^7, 6×10^7, and 2.5×10^7 organisms, respectively. In one group

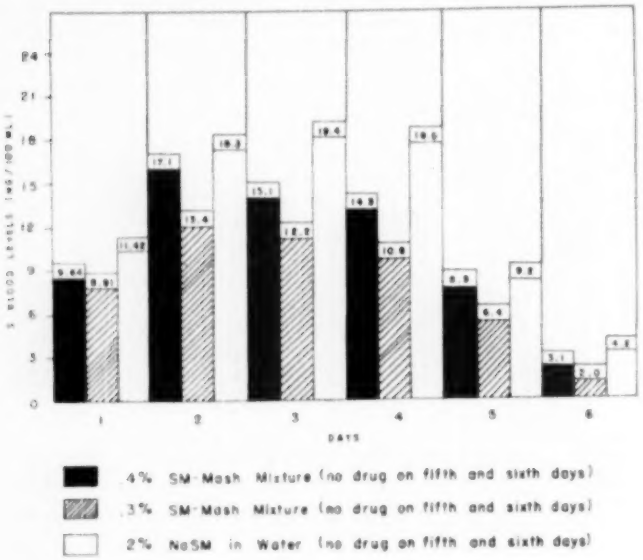


Fig. 2—Blood-sulfamerazine levels in chickens during a period of six days.

that was exposed to 2.5×10^8 organisms, the ration was supplemented by the addition of 4,500 units of cod liver oil per pound of mash and alfalfa leaf meal (20% protein) at a level of 1 lb. to 3 lb. of mash for three weeks before reëxposure. All the previously exposed birds became sick; however, only 1 turkey died in each group that received 2.5×10^8 and 6×10^7 organisms and 2 turkeys died in the group that received 8×10^7 organisms. All exposed, susceptible control turkeys, 2 in each group, died within forty-eight hours.

Nine weeks after the first reëxposure to *P. multocida*, or three and one-half months after the initial exposure to the organism, 18 turkeys were separated into three groups of 6 birds each, and reëxposed a second time with 10×10^7, 5×10^7, and 5×10^6 intramuscular doses, respectively, of strain 6-7. None of the previously exposed birds died; however, 1 turkey that had received 10×10^7 organisms became emaciated and a pectoral abscess was found near the site of inoculation. This bird was killed and *P. multocida* was isolated from the abscessed tissue. All exposed, susceptible turkeys, 2 in each group, died within forty-eight hours. Sulfamerazine was not employed during the first or second reëxposure tests.

Experiment E—Sulfamerazine Treatment of Turkeys During Field Outbreak of Fowl Cholera.—In a previous paper, Alberts and Graham[9] reported that a 0.5 per cent sulfamerazine-mash mixture reduced losses due to fowl cholera in a flock of 1,960 tom turkeys during the period of treatment and for at least two days after treatment was stopped. The disease reappeared twice during the observation period; in each instance, losses subsided after an additional course of sulfamerazine therapy.

Following this report, sporadic outbreaks of fowl cholera have been observed in three Illinois turkey flocks.

An affected 6-month-old flock was placed on a 0.5 per cent sulfamerazine-mash mixture for four days and marketed immediately. A slight lack in quality of these birds caused some loss in profit. The mortality was 4.1 per cent.

In the second outbreak among 5-month-old turkeys, 0.2 per cent sodium sulfamerazine was added to the drinking water on the first, second, third, fifth, eighth, and twelfth days. The disease recurred the twenty-ninth day after treatment was stopped. Another three-day course of therapy was administered, and the birds were marketed. A loss of 7.4 per cent of the turkeys was attributed to fowl cholera.

In a 4-month-old flock, the disease was highly contagious and its course rapid. Losses were 21.5 per cent before sulfamerazine therapy could be administered. The weather was below freezing and windy. A 0.4 per cent sulfamerazine-mash mixture was given, but feed consumption was less than normal. Not until the seventh day of treatment did losses subside. The disease reappeared on the fourth day after treatment was stopped and intermittent sulfamerazine therapy was begun. A 0.4 per cent sulfamerazine-mash mixture was provided on the first, second, third, fifth, eighth, and twelfth days. The disease reappeared on the seventh day after the intermittent course of treatment was stopped. Sulfamerazine was given for three days, and the remaining, apparently healthy birds were sold at a sharply reduced price. From the beginning of sulfamerazine therapy until the birds were sold, 19.7 per cent of the flock died.

TABLE 1—Experiment A—Effect of Sodium Sulfamerazine After Simultaneous Treatment and Exposure to a Lethal Dose of *Pasteurella Multocida* Strain 6-7 in Turkeys

No. of turkeys	Exposure dose of organisms intramuscularly	Drug* intake mg./kg./day	Response			
			Five days		Thirty days	
			Alive	%	Alive	%
30	2×10^8	143.00	30	100	25	83
15	2×10^8	107.25	13	87	10	66
15	2×10^8	71.5	10	66	3	20
10	2×10^8	35.75	4	40	1	10
10	2×10^8	0.00	0	0	...	...

*Drug administered orally twice daily for two days.

DISCUSSION

The results of experiment A indicate that

143.0 and 107.25 mg. of sodium sulfamerazine per kilogram of body weight protects turkeys against experimentally induced fowl cholera during the period of treatment and for two to four days after treatment is stopped. Two lower dosage levels, 71.5 or 35.75 mg. per kilogram of body weight, are not as effective. The reappearance of the disease emphasized the importance of carrier birds, which was demonstrated by the isolation of the organism from a lung abscess of 1 of the birds. Reis[10] recovered *P. multocida* just before death from the feces of birds experimentally infected by the intramuscular or nasal routes, and from the nasal mucus early in the course of the disease. Furthermore, Reis[11] has shown that "healthy carriers" may be found among chickens that received simultaneous injection of virulent *P. multocida* and sulfanilamide.

TABLE 2—Experiment A—Effect of Sulfamerazine and Sodium Sulfamerazine Before and After Intramuscular Exposure to *Pasteurella Multocida* Strain 6-7 in Chickens

No. of birds	Drug (%)	Exposure dose of organisms intramuscularly	Response			
			Five days		Thirty days	
			Alive	%	Alive	%
20	0.4*	2×10^8	17	85	12	60
20	0.3*	2×10^8	15	75	11	55
10	0.2*	2×10^8	6	60	3	30
20	0.2**	2×10^8	17	85	14	70
20	0.1**	2×10^8	12	60	10	50
20	none	2×10^8	9	45	5	20

*Treated birds had access to the sulfamerazine-mash mixture two days before and five days after exposure to strain 6-7.
**Treated birds had access to sulfamerazine in the drinking water two days before and five days after exposure to strain 6-7.

Losses were reduced among chickens that had access to either 0.4 or 0.3 per cent sulfamerazine in the mash for five days after exposure to *P. multocida*. A level of 0.2 per cent of the sulfonamide-mash mixture was ineffective. Twenty-five days after treatment was discontinued, losses were 50.0 and 43.7 per cent less in chickens that had received either 0.4 or 0.3 per cent, respectively, of the sulfonamide-mash mixture. After treatment was discontinued, the disease reappeared in only 1 or 2 chickens at any one time. In protecting chickens exposed to *P. multocida*, 0.1 and 0.2 per cent sodium sulfamerazine in the drinking water were about as effective as 0.3 and 0.4 per cent sulfamerazine in the mash.

Since fewer untreated, exposed chickens died during the initial five days after intranasal exposure to *P. multocida*, the reduction in losses among birds that received either sulfamerazine or sodium sulfamerazine was not particularly striking. However, during the twenty-five days after treatment was stopped, losses were reduced 56 and 59 per cent among chickens that had received 0.4 per cent sulfamerazine or 0.2 per cent sodium sulfamerazine, respectively.

Although the number of birds was small, blood-sulfamerazine levels recorded in experiment B (fig. 1) suggest that more than 5 mg. per 100 ml. of blood may be required to prevent death of turkeys exposed to *P. multocida*. The blood levels of 3 turkeys which died were less than 5 mg. per 100 ml. of blood. Extremes above and below the mean blood-sulfamerazine levels were observed in different turkeys of the same group that received a uniform dosage of the

TABLE 3—Experiment A—Effect of Sulfamerazine and Sodium Sulfamerazine Before and After Nasal Instillation of *Pasteurella Multocida* Strain 6-7 in Chickens

No. of birds	Drug* (%)	Exposure dose of organisms	Response			
			Five days		Thirty days	
			Alive	%	Alive	%
20	0.4 in mash	2×10^8	19	95	17**†	85
20	0.2 in water	2×10^8	20	100	16	80
20	None	2×10^8	14	70	7**†	35

*Treated chickens received sulfamerazine in the mash or sodium sulfamerazine in the drinking water for a period of five days after exposure to *Pasteurella multocida*.
**Carriers; † = abscess, ‡ = nasal cleft.

[10] Reis, E. A. G.: Studies on the Pathogenesis of Fowl Cholera. I. Experimental Infection of Chickens with *Pasteurella Multocida*. Am. J. Vet. Res. 5:1-6, 1944.

[11] Reis, E. A. G.: Studies on the Pathogenesis of Fowl Cholera. II. Experimental Infection of Chickens with *Pasteurella Multocida* and Sulfanilamide. Am. J. Vet. Res. 5:7-11, 1944.

drug based on weight. These differences may have been due either to differences in amounts of sulfamerazine absorbed from the intestinal tract or to differences in rates of excretion of the drug by the kidney.

Chickens that had access to 0.4 or 0.3 per cent sulfamerazine in the mash or 0.2 per cent sodium sulfamerazine in the drinking water appeared to develop adequate blood-sulfonamide levels (fig. 2). The higher mortality among the chickens that received either 0.2 per cent sulfamerazine in the mash or 0.1 per cent sodium sulfamerazine in the drinking water appears to be due to an insufficient amount of the drug.

In experiment C, sulfamerazine and sodium sulfamerazine were not immediately effective in reducing mortality in chickens after experimentally induced fowl cholera had become established. A number of the birds were visibly sick at the time treatment was begun. Since the effectiveness of the sulfonamide depends upon adequate consumption of the drug, it is not unreasonable to assume that increased losses occurring during the period of treatment were the result of widespread tissue damage before the drug could exert its optimum antibacterial activity.

The results reported in experiment D suggest that turkeys which recover from *P. multocida* infection after sulfamerazine therapy may have developed some immunity against subsequent infections. These findings further suggest that sulfamerazine may not impair the development of immunity. There is evidence that the acquired resistance may last two months.

orally for two days at the rate of 143.0 and 107.25 mg. per kilogram of body weight effectively reduced mortality in turkeys due to experimentally induced fowl cholera. After treatment ceased, the disease reappeared, but was again controlled by oral sodium sulfamerazine therapy.

2) There were 72.7 and 54.5 per cent fewer losses in chickens receiving either a 0.4 or 0.3 per cent sulfamerazine-mash mixture, respectively, than in untreated control birds. The addition of 0.2 per cent sodium sulfamerazine to the drinking water protected *Pasteurella multocida*-infected chickens to about the same extent as 0.4 per cent sulfamerazine in the mash.

TABLE 4—Experiment C—Therapeutic Effect of Sulfamerazine and Sodium Sulfamerazine in Experimentally Induced Fowl Cholera of Chickens

No. of birds	Exposure dose of strain 6-7 intramuscular	Drug (%)	Response					
			Alive		Alive		Alive	
			2 days	%	7 days	%	30 days	%
20	1.5×10^8	0.4% SM in mash 3rd thru 7th day after exposure.	17	85	11	64	7	41
20	1.5×10^8	0.2% NaSM in water 3rd thru 7th day after exposure.	16	80	13	81	10	62
10	1.5×10^8	None	8	80	4	50	2	25

SM = sulfamerazine; NaSM = sodium sulfamerazine.

3) Differences in blood-sulfamerazine levels were observed in turkeys that received the same amount of the drug per unit of weight, as well as in chickens that had access to the same amounts of the drug in mash or water.

4) Sulfamerazine and sodium sulfamerazine reduced losses in chickens after experimentally induced fowl cholera had become established. The greatest losses occurred during the two days after treatment was started.

5) Turkeys that had recovered from an experimental *P. multocida* infection and treatment with sodium sulfamerazine were partially immune five weeks later to a challenge reexposure with the organism. They were even more resistant to a second reexposure nine weeks later.

SUMMARY

1) Sodium sulfamerazine administered

References

[1]Levaditi, C., and Reinie, L.: Chimiotherapie de l'Infection Determinee Chez la Souris et la Poule par la *Pasteurella avicida*. Compt. rend. Soc. de Biol., 127: (1938) : 1178.

[2]Cardoso, L. M., Nobrega, P., and Reis, J.: Cacao

da Sulfanilamida Sobre a Infeccao por *Pasteurella Avicida* Em Aves. Arq. Inst. Biologico, São Paulo, Brazil, **9**, (1938) :253.

[5]DeVolt, H. M.: Sulfathiazole as an Aid in the Control of Fowl Cholera in Chickens and Turkeys. Am. J. Vet. Res., **9**, (1948) : 215.

[6]Kiser, J. S., Prier, J. E., Botterff, C. A., and Greene, L. M.: Treatment of Experimental and Naturally Occurring Fowl Cholera with Sulfamethazine. Poult. Sci., **27**, (1948) : 257.

[7]Hilbert, K. F., and Kiser, J. S.: Chemotherapy of Duck Diseases with Sulfonamides. Cornell Vet., **38**, (1948) : 148.

[8]Hughes, T. P.: The Epidemiology of Fowl Cholera. II. Biological Properties of *Pasteurella Avicida*. J. Exptl. Med., **51**, (1930) : 225.

[9]Raper, K. B., and Alexander, D. F.: Preservation of Molds by the Lyophil Process. Mycologia, **37**, (1945) : 499.

[10]Bratton, A. C., and Marshall, E. K.: A New Coupling Component For Sulfanilamide Determination. J. Biol. Chem., **128**, (1939) : 537.

[11]Alberts, J. O., and Graham, R.: Sulfamerazine in the Treatment of Fowl Cholera in Turkeys. Am. J. Vet. Res., **9**, (1948) : 310.

[12]Reis, J.: Eliminacao da *Pasteurella Avicida* Pelas Fezes. Arq. Inst. Biologico, São Paulo, Brazil, **12**, (1941) : 307.

[13]Reis, J.: Duracao de Immunidade Conferida por Injecoes Simultaneous de *Pasteurella Avicida* e Sulfanilamida. Arq. Inst. Biologico, São Paulo, Brazil, **10**, (1939) : 301.

The Prophylactic and Therapeutic Properties of Sulfamerazine in Fowl Typhoid

J. O. ALBERTS, V.M.D., Ph.D.

Urbana, Illinois

THE REPORTS made by Severens, Roberts, and Card,[1] and Bottorff and Kiser[2] on the effectiveness of sulfamerazine on preventing losses in chickens exposed to *Salmonella pullorum* suggested the possibility that this drug might be effective in preventing losses caused by *Salmonella gallinarum*. Both organisms are similar in morphologic and antigenic structure and are transmitted naturally in a similar manner.

Holtman and Fisher[3, 4] found sulfamerazine and sodium sulfamerazine highly efficient in reducing mortality due to experimentally induced fowl typhoid of young chickens. Recurrences of the disease were noted, but on each occasion losses stopped after additional treatment with the drug. According to Pomeroy, Fenstermacher, and Roepke,[5] sulfamerazine did not protect chicks exposed to *S. gallinarum*. Because of differences in the results of these workers, it was considered worthwhile to study further the preventive and therapeutic effects of sulfamerazine on *S. gallinarum* infections.

MATERIALS AND METHODS

Three-day-old New Hampshire Red chicks were used in exposure studies of fowl typhoid. *Salmonella gallinarum* strain 10-4, which was used in these experiments, had been isolated from chickens that died during an outbreak of acute fowl typhoid. Lyophilized pure cultures were prepared by a slight modification of the method of Raper and Alexander.[6]

Day-old chicks, from pullorum-free flocks, were kept in electric brooders and fed a starter mash of the same composition as that used by the University of Illinois Division of Poultry Husbandry.

At 3 days of age, chicks were exposed orally with 7.5×10^7 to 1.25×10^8 *S. gallinarum*. Sulfamerazine and sodium sulfamerazine in different quantities were added to the mash or drinking water for varying periods before or after exposure to the organisms. Exposed and unexposed, untreated chicks were included in each experiment as controls. Blood-sulfamerazine levels were determined according to the method of Bratton and Marshall[7] and readings were made with a Klett-Summerson colorimeter.

EXPERIMENTAL RESULTS

Experiment A — Prophylactic Action of Sulfamerazine Against Experimental Fowl Typhoid in Chickens.—Losses were almost entirely prevented in 3-day-old chickens that received either 0.4 per cent sulfamerazine-mash mixture or 0.2 per cent sodium sulfamerazine in the drinking water for seven consecutive days immediately after exposure to *S. gallinarum* (table 1). No treatment was given on the following fourteen days. Three weeks after exposure to the organisms, mortality was 28.0 and 33.4 per cent less in the treated groups of birds. During the two weeks after treatment was discontinued, however, the recurrence of fowl typhoid caused a loss of 67.0 and 60.0 per cent in the chicks that had received sulfamerazine.

The results of the intermittent use of sulfamerazine and sodium sulfamerazine following exposure of chicks to *S. gallinarum* are shown in table 2. The birds had access to mash or water containing the sulfonamide on the first, second, fourth, fifth, eighth, twelfth, and seventeenth days after exposure. Three weeks after exposure to the organism, mortality was reduced 70 and 67 per cent in chicks that had access to 0.5 or 0.4 per cent sulfamerazine-mash mixtures. Losses were 75 per cent less in chicks that received 0.2 per cent sodium sulfamerazine in the drinking water than in exposed, untreated chicks. At the end of twenty-one days, losses were 53 per cent less among birds that had access to 0.3 per cent sulfamerazine in the mash, and 32 per cent less in chicks that had access to 0.1 per cent sodium sulfamerazine in the drinking water than in exposed, untreated chicks.

Experiment B — Blood-Sulfamerazine

A section from a thesis submitted in partial fulfillment of the requirements for the degree of doctor of philosophy in the Graduate College of the University of Illinois.

From the Department of Veterinary Pathology and Hygiene, College of Veterinary Medicine and Agricultural Experiment Station, University of Illinois, Urbana.

Levels in Chickens Exposed to Salmonella Gallinarum.—Blood-sulfamerazine determinations were made each day on 5 different chicks from four groups of 30 each that had received either 0.4 or 0.3 per cent sulfamerazine-mash mixtures or 0.2 or 0.1 per cent, respectively, sodium sulfamerazine in the drinking water. The sulfonamide-mash or water mixtures were kept before the birds for three days. The first day after sulfamerazine therapy was begun, all chicks were exposed to the organism. Results of the blood-sulfamerazine determinations of the different sulfonamide dosage levels are shown in fig. 1. Residual blood-sulfamerazine levels were determined on the first and second days after therapy was discontinued.

Experiment C—Therapeutic Action of Sulfamerazine in Experimentally Induced Fowl Typhoid of Chickens.—Sulfamerazine therapy was delayed until the fifth day after exposure to the organism. At this time, a few chicks in each exposed group had died and several birds appeared listless. The results are presented in table 3.

TABLE 2—Experiment A—Effect of Sulfamerazine and Sodium Sulfamerazine with Intermittent Treatment of Chicks Exposed to *Salmonella Gallinarum*

No. of chicks	Drug* (%)	Exposure	Chicks alive after 21 days	%
60	0.5% in the mash	1.25×10^8	42	70
60	0.4% in the mash	1.25×10^8	40	67
60	0.3% in the mash	1.25×10^8	32	53
60	0.2% in the water	1.25×10^8	45	75
60	0.1% in the water	1.25×10^8		
60	None	1.25×10^8	0	0
60	None	None	60	100

*Birds had access to sulfamerazine in the mash or sodium sulfamerazine in the drinking water on the first, second, fourth, fifth, eighth, twelfth, and seventeenth days after exposure.

A total of 25 (48%) chicks died during the seven-day period of sulfamerazine therapy, of which 14 died during the first two days after treatment was begun. A similar reaction was noted in the chicks that received sodium sulfamerazine; 30 (49%) birds died, but 25 of the chicks succumbed within two days after treatment was started.

TABLE 1—Experiment A—Effect of Sulfamerazine and Sodium Sulfamerazine in Experimentally Induced Fowl Typhoid in Chicks

No. of chicks	Drug* (%)	Exposure dose of organism	No. of chicks alive			
			7 days	%	21 days	%
60	0.4% in mash	1×10^8	60	100	20	33
60	0.2% in water	1×10^8	59	98	22	38
40	None	1×10^8	17	42	3	7
40	None	None	38**	95	38	95

*Treated chicks received sulfamerazine in mash or sodium sulfamerazine in the drinking water for seven days after exposure to *Salmonella gallinarum*.

**Two birds died; cause of death not known.

Experiment D—Recurrences of Fowl Typhoid in Chickens.—Throughout the experimental observations of induced fowl typhoid in chickens, reappearance of the disease was observed after sulfamerazine medications were discontinued. Similar results have been observed by Holtman and Fisher,[4] and Hall *et al.*[5] Such recurrence was more evident following the continuous use of the drug for five to eight days than after the intermittent use of the drug. The cumulative mortality in young chicks caused by fowl typhoid after sodium sulfamerazine therapy was discontinued is shown in table 4. Sodium sulfamerazine therapy was not begun until the fourth day after 105, 3-day-old chicks were exposed orally to approximately 1×10^8 viable organisms of strain 10-4. Two chicks died during the three days before treatment was begun, and *S. gallinarum* was isolated from the liver and heart of 1 of the birds. On the sixth and ninth days after exposure, the birds were transferred to clean, disinfected brooders. During the eight-day course of sodium sulfamerazine therapy, 16 (15.5%) chicks died. A total of 31 (30.1%) chicks died within thirteen days after treatment was stopped.

An attempt was made to determine whether *S. gallinarum* remained in the tissues of exposed chicks that had continuous access to sodium sulfamerazine or sulfamerazine in the water or mash. Three

days after oral exposure to 7.5×10^7 organism, 250 chicks were separated into three groups of 100, 100, and 50 birds each. One group of 100 chicks received mash containing 0.4 per cent sulfamerazine, which was kept before the birds for twenty-one days. A similar group of 100 chicks had access to drinking water containing 0.2 per cent sodium sulfamerazine. The remaining 50 exposed chicks served as untreated controls.

In the group of 100 exposed chickens that had access to a 0.4 per cent sulfamerazine-mash mixture for twenty-one days, 85 birds were killed during four-day intervals while still on treatment. *Salmonella gallinarum* was isolated from the hearts and livers of 48 (56.4%) chickens. At necropsy, macroscopic lesions suggestive of fowl typhoid were observed in 24 (28.2%) of the chickens. Similarly, in a group of 100 exposed chickens that had access to drinking water containing 0.2 per cent sodium sulfamerazine, 90 birds were killed during four-day intervals while still on treatment. *Salmonella gallinarum* was isolated from the livers and hearts of 37 (41.1%) chicks. At necropsy, macroscopic lesions suggestive of fowl typhoid were observed in 17 (18.9%) of the chickens.

Approximately 2 ml. of heart blood was collected aseptically from each of 10 chickens on the twenty-first day after they had continuously received either 0.4 per cent sulfamerazine in the mash or 0.2 per cent sodium sulfamerazine in the drinking water. One of ten blood cultures from chickens that received sulfamerazine contained *S. gallinarum*, while none of ten blood cultures from chickens that received sodium sulfamerazine contained the organism.

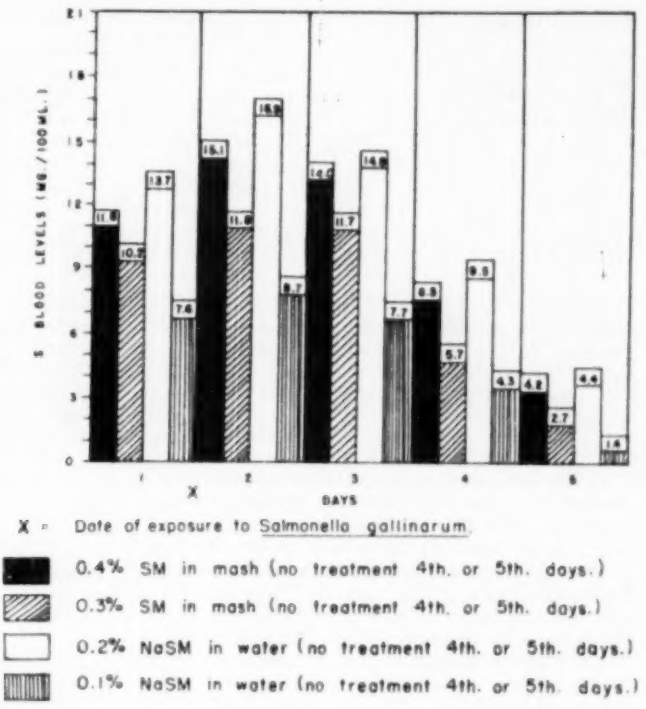


Fig. 1 — Blood-sulfamerazine levels in chicks exposed to *Salmonella gallinarum*.

Experiment E—Use of Sulfamerazine in a Sporadic Outbreak of Fowl Typhoid in Turkeys.—Fowl typhoid appeared in a group of 370, 12-week-old turkeys. A group of 270 birds received a 0.5

per cent sulfamerazine-mash mixture for seven successive days. Another group of 100 turkeys was maintained as untreated controls. Losses in the treated turkeys were reduced 78.2 per cent at the end of seven days. Unfortunately, post-treatment observation of the treated and untreated birds was not possible, since the flock caretaker placed these poults on range with another group of 1,100 poults of the same age. The flock owner later reported that more than 100 poults died, presumably from fowl typhoid.

TABLE 3—Experiment C—Therapeutic Effect of Sulfamerazine and Sodium Sulfamerazine in Experimentally Induced Fowl Typhoid of Chicks

No. of chicks	Exposure dose of strain 10-4 per os	Drug* (%)	No. of chicks alive					
			4 days	%	11 days	%	30 days	%
60	1 x 10⁸	0.4% SM** in mash	52	87	27	52	21	40
60	1 x 10⁸	0.2% NaSM** in water	58	97	28	48	19	33
70	1 x 10⁸	None	28	93	2	7	0	0
40	None	None	29**	97	29	97	39	97

*Treatment began the fifth day and ended the eleventh day after exposure.
**One chick died, cause of death not known. SM = sulfamerazine; NaSM = sodium sulfamerazine.

DISCUSSION

Chicks were protected against an experimental *S. gallinarum* infection during a seven-day period of continuous treatment with 0.4 per cent sulfamerazine in the mash or 0.2 per cent sodium sulfamerazine in the drinking water. This method of therapy, however, cannot be considered satisfactory, since 60 to 65 per cent of the chicks died within two weeks after it was discontinued. These results suggest that sulfamerazine did not destroy all *S. gallinarum* in the body of exposed birds.

The intermittent use of 0.5 per cent sulfamerazine in the mash or 0.2 per cent sodium sulfamerazine in the drinking water, *i.e.*, on the first, second, fourth, fifth, eighth, twelfth, and seventeenth day after exposure to the organisms, was effective in reducing losses caused by *S. gallinarum*. When the same amount of drug was given over a longer period, losses following the recurrence of fowl typhoid were reduced about 60 per cent by the intermittent method as compared to the continuous method of treatment. Further study on the intermittent method of treatment for fowl typhoid seems worthwhile, particularly in outbreaks of disease among birds in broiler establishments. The intermittent use of a 0.3 per cent sulfamerazine-mash mixture or 0.1 per cent sodium sulfamerazine in the drinking water did not provide as good protection as 0.4 per cent sulfamerazine and 0.2 per cent sodium sulfamerazine against fowl typhoid at the end of twenty-one days.

Chicks that had access to either 0.4 or 0.3 per cent sulfamerazine in the mash or 0.2 or 0.1 per cent sodium sulfamerazine in the drinking water (fig. 1) developed a mean blood-sulfamerazine level of 13.6, 11.2, 15.1, and 8.0 mg. per 100 ml. of blood, respectively, during the three-day period of treatment. The mean blood-sulfonamide level did not fall below 5 mg. per 100 ml. of blood until the second day after treatment was discontinued, except in chicks that received 0.1 per cent sodium sulfamerazine in the drinking water.

Sulfamerazine and sodium sulfamerazine

TABLE 4—Experiment D—Recurrence of Fowl Typhoid in Chicks after Eight Days Sodium Sulfamerazine Therapy

No. of chicks	Date	Number died	Cumulative mortality (%)	Treatment*
103	7-18	4	3.9	+
	7-19	2	5.8	+
	7-20**	2	7.7	+
	7-21	6	13.6	+
	7-22	2	15.5	+
	7-23**	0	15.5	+
	7-24	0	15.5	+
	7-25	0	15.5	+
	7-26	0	15.5	—
	7-27	0	15.5	—
	7-28	3	17.4	—
	7-29	4	21.3	—
	7-30	6	27.1	—
	7-31	4	31.0	—
	8-1	7	37.8	—
	8-2	3	40.7	—
	8-3	1	41.7	—
	8-4	0	41.7	—
	8-5	2	43.6	—
	8-6	0	43.6	—
	8-7	2	45.6	—

*0.2% sodium sulfamerazine in the drinking water.
**Birds transferred to a clean and disinfected brooder.

were effective in checking losses in chicks within five days after experimentally induced fowl typhoid had become established. The fact that the sulfonamide does not exert an immediate antibacterial action is indicated by the deaths of 36 and 43 per cent of the chicks that received sulfamerazine and sodium sulfamerazine, respectively, within two days after treatment was started.

In experiment D, the reappearance of fowl typhoid in chickens that had been treated with 0.2 per cent sodium sulfamerazine may have been caused by contamination of the screen platform, water, and feed trays by *S. gallinarum* from excretions of exposed birds. Although the treated birds were transferred to clean and disinfected brooders on the third and sixth days of treatment, this control measure did not exclude the possibility that the organism might have been eliminated in the feces of the treated birds after the second transfer to clean and disinfected surroundings. It should be noted that losses ceased during the last three days of treatment and did not reappear until the third day after treatment was discontinued.

In birds killed after receiving either drug for one to twenty-one days, 22 colonies of *S. gallinarum* was the largest number isolated from the liver or heart cultures of any bird. Cultures from a majority of the birds on continuous treatment contained less than ten colonies of the organism. On the other hand, the colonies of *S. gallinarum* were so numerous that an accurate count was not possible from liver and heart cultures of chickens sacrificed three days after being taken off treatment. Although macroscopic lesions were observed in a number of the chickens killed during the period of treatment, these tissue changes did not seem to impair noticeably the living birds' activities.

SUMMARY

1) A 0.4 per cent sulfamerazine-mash mixture or 0.2 per cent sodium sulfamerazine in the drinking water was effective in preventing losses caused by *Salmonella gallinarum* in chicks, during a seven-day course of treatment and for two days after treatment was stopped. The disease recurred and losses were often severe unless an additional course of treatment was given.

2) The intermittent use of sulfamerazine or sodium sulfamerazine during a 21-day period protected more chicks exposed to *S. gallinarum* than the use of the same levels of the drug for seven successive days.

3) Chicks that received 0.4 or 0.3 per cent sulfamerazine in the mash and 0.2 or 0.1 per cent sodium sulfamerazine in the drinking water developed mean blood-sulfamerazine levels of 13.6, 11.2, 15.1, and 8.0 mg. per 100 ml. of blood, respectively, during a three-day period of treatment.

4) Losses were severe during the first two days in chicks that received sulfamerazine or sodium sulfamerazine after induced fowl typhoid had become established. However, losses stopped on the fifth day of treatment.

5) *Salmonella gallinarum* was isolated from the liver and heart cultures of 56.4 and 41.1 per cent of the chicks killed at intervals during twenty-one days of continuous sulfamerazine and sodium sulfamerazine treatment.

6) The use of 0.5 per cent sulfamerazine in the mash for one week reduced losses 78.2 per cent in turkey poults during a natural outbreak of fowl typhoid.

References

[1]Severens, J. M., Roberts, E., and Card, L. E.: The Effects of Sulfonamides in Reducing Mortality from Pullorum Disease in Domestic Fowls. Poult. Sci., 24, (1945):155.

[2]Bottorff, C. A., and Kiser, J. S.: The Use of Sulfonamides in the Control of Pullorum Disease. Poult. Sci., 26, (1947):355.

[3]Holtman, D. F., and Fisher, G.: Sulfa Drugs in the Control of *Shigella Gallinarum* Infection. J. Bact., 51, (1946):589.

[4]Holtman, D. F., and Fisher, G.: The Application of Sulfonamides in the Control of Fowl Typhoid in Poultry. Poult. Sci., 26, (1947):478.

[5]Pomeroy, B. S., Fenstermacher, R., and Roepke, M. H.: Sulfonamides in the Control of Salmonelloses of Chicks and Poults. J.A.V.M.A., *112*, (1948):296.

[6]Raper, K. B., and Alexander, D. F.: Preservation of Molds by the Lyophil Process. Mycologia, *37*, (1945):499.

[7]Bratton, A. C., and Marshall, E. K.: A New Coupling Component for Sulfanilamide Determination. J. Biol. Chem., *128*, (1939):537.

[8]Hall, W. J., MacDonald, A. D., and Legenhausen, D. H.: Studies on Fowl Typhoid. II. Control of the Disease. Poult. Sci., 28, (1949):789.

Search for Virus in Eggs Laid During Recovery Postinoculation with Newcastle Disease Virus

J. A. BIVINS, D.V.M., BARBARA RHODES MILLER, B.S., and F. R. BEAUDETTE, D.V.M.

New Brunswick, New Jersey

THERE ARE a few reports of the occurrence of Newcastle disease virus (NDV) in hen eggs.

LITERATURE CITED

Van Roekel[1] and Jungherr[2] both refer to its occurrence in fresh eggs. In Jungherr's case, these were fresh eggs obtained about two months after chicks put out by the hatchery receiving the eggs developed Newcastle disease (ND). DeLay[3] reported the isolation of NDV from the yolk sacs of 6 chicks, 4 days old, which were hatched from eggs collected while production was depressed from ND. The virus was also obtained from dead embryos in the succeeding hatch, the eggs for which were collected when the flock was recovering and increasing in production. Infertile eggs from another flock collected prior to, during, and following the period of clinical illness yielded NDV, but no virus was found in 20 live embryos from the same setting. Massachusetts workers[4] studied the occurrence of NDV for thirty-three days following inoculation of 18 pullets, 7 months old. Of 250 eggs which were cultured, virus was found only in three eggs laid on the fifth day after inoculation, in one on the sixth, and in two on the seventh. Beaudette *et al.*[5] reported the isolation of NDV from three of ten oviduct eggs examined. Thompson and Osteen[6] recovered virus from three of 85 yolk samples examined from a flock affected with a natural outbreak of ND. The recoveries were from eggs laid at the beginning of the outbreak. Hofstad,[7] in a study involving three flocks, found the virus present only in eggs laid during the decline in production. A total of 837 eggs, collected from three different flocks, as production began to increase from its lowest point, failed to yield a single positive NDV isolation. Beaudette[8] has referred to the inoculation of a laying flock and the recovery of virus from eggs laid during the period of systemic reaction but not after recovery. We present more data on that case.

EXPERIMENTAL

A hatcheryman whose flock had had ND the previous year requested that his replacement flock be inoculated so that parentally immune chicks could be obtained the following spring. Accordingly, a relatively avirulent strain of NDV (Mathews-spleen; not Roakin-spleen 2, the strain presently used) was inoculated into 10-day chicken embryos and allantoic fluid collected when the embryos died. A 2 per cent saline suspension of allantoic fluid was prepared and each bird was inoculated with 0.2 cc. in the pectoral muscles. The birds were housed in four different buildings as follows: house 1, 2,000 Leghorns; house 2, 1,600 Leghorns; house 3, 200 Rhode Island Reds; house 4, 400 mixed birds. Birds in houses 1, 2, and 3 were inoculated on Nov. 14, 1947; those in house 4 on Nov. 18, 1947. Production records for the two larger houses were kept for two and one-half months following inoculation. Table 1 shows the trend of production.

TABLE 1—Trend of Production in Birds Injected with Newcastle Disease Virus of Low Virulence

Date (1947)	Prod.	Date (1947)	Prod.	Date (1947–1948)	Prod.
11/14	2600	12/1	128	12/29/47	1907
11/17	2171	12/8	478	1/ 7/48	2071
11/22	15	12/15	825	1/18	2187
11/23 to 28	10*	12/22	1432	1/30	2263

*Average daily production.

A few floor eggs were collected from birds in house 4 on the fourth, fifth, and sixth days postinoculation. Individual yolk samples were collected without contamination and inoculated into 10-day chicken embryos. The virus was recovered from none of seven eggs collected on the fourth day postinoculation, from four of five eggs on the fifth day, and not from the only egg collected on the sixth day.

To study the problem further, 20 eggs from a pen which returned to production

Paper of the Journal series, New Jersey Agricultural Experiment Station, Department of Poultry Husbandry, Rutgers University, New Brunswick, N. J.

Since preparation of this manuscript, we have noted an article by Illinois workers (Prier *et al.*) in which similar results were obtained.

more slowly than the rest of the flock were set in an incubator approximately twice each week between the twenty-seventh and seventy-seventh days postinoculation. These eggs were candled for dead embryos daily after the third or fourth days of incubation. Table 2 shows the disposition of these eggs.

TABLE 2—Disposition of Incubated Eggs

Contaminated, not used for inoculation	16
Broken, not used for inoculation	3
Used as inoculum, not contaminated	96
Used as inoculum, but contaminated	4
Inoculum not saved	5
Pipped only, not examined	5
Pipped, dead, not examined	155
Hatched	0

Yolk was saved from each infertile egg, as was tissue (usually liver) from each embryo which died before twenty-one days of incubation. A total of 100 such samples was inoculated into chicken embryos, of which 4 were contaminated with bacteria. These 4 were inoculated on the day of harvest; consequently, contamination was not detected until later. Of the remaining 96, virus was not recovered from a single specimen.

We cannot but agree with Hofstad[6] when he says, "It is difficult to believe that true egg transmission can occur in Newcastle disease."

References

[1]Van Roekel, H.: Proc. Conference Newcastle Disease, U. S. D. A., (May, 1946) 17.

[2]Jungherr, E.: Proc. Conference Newcastle Disease, U. S. D. A., (May, 1946) 56, 93.

[3]De Lay, P. D.: Isolation of Avian Pneumoencephalitis (Newcastle Disease) Virus from the Yolk Sac of Four-Day-Old Chicks, Embryos, and Infertile Eggs. *Science, 106*, (1947): 545-546.

[4]Anonymous, Dept. of Veterinary Science, Univ. of Massachusetts: Newcastle Disease Studies. Proc. Twentieth Ann. Meet. Northeastern Conference of Laboratory Workers in Pullorum Disease Control. Richmond, Va., June 8-10, 1948.

[5]Beaudette, F. R., Bivins, J. A., Miller, Barbara Rhodes, Hudson, C. B., and Black, J. J.: Studies on the Diagnosis of Newcastle Disease in New Jersey. Am. J. Vet. Res., 9, (1948) :69-76

[6]Thompson, C. H., and Osteen, O. L.: A Technique for the Isolation of Newcastle Disease Virus, Using Streptomycin as a Bacterial Inhibitor. Am. J. Vet. Res., 9, (1948) :303-305.

[7]Hofstad, M. S.: A Study on the Epizootiology of Newcastle Disease (*Pneumoencephalitis*). Poult. Sci., 28, (1949) :530-533.

[8]Beaudette, F. R.: The Immunization of Birds Against Newcastle Disease. Proc. Fifty-second Ann. Meet. U. S. Livestock Sanitary A., Denver, Colo., (Dec., 1948) :254-265.

Studies in Avian Leucosis. V. An Investigation of the Possible Relationship of Sex Hormones to Visceral Lymphomatosis

OLIVE STULL DAVIS, Ph.D., F. N. ANDREWS, Ph.D., and L. P. DOYLE, D.V.M., Ph.D.

Lafayette, Indiana

IT HAS LONG been known that certain types of neoplasms are associated with the genital and mammary systems. It is likewise known that hormones, particularly some of the estrogens, may be carcinogenic. In recent years, evidence has been accumulating suggesting that the endocrine system and certain hormones may affect both the development and the regression of some neoplastic growths.

In several recent papers, Gardner and his coworkers[6, 7, 8] and Silberberg and Silberberg[11] have shown a relationship between sex hormones and leucemia in mice.

In chickens, the incidence of leucosis has been reported by Marine and Rosen,[9, 10] Burmester,[1] Burmester and Nelson,[2] and Davis and Doyle[3] to be higher in females than in males. Furthermore, the incidence of both spontaneous and experimentally produced avian lymphomatosis was observed by all these authors to be higher in castrated males than in normal males.

A fuller investigation of the possible relationships between sex hormones and avian visceral lymphomatosis was undertaken in two transmission experiments, both of which are described here.

SEX HORMONE EXPERIMENT I

A preliminary study was made in connection with the transmission experiment conducted at this laboratory in 1944 to 1945, primarily concerned with transmission of visceral lymphomatosis by the use of various affected organs as inoculums, and previously described.[5]

Experimental Procedure.—On Nov. 3, 1944, 300 Barred Plymouth Rock female chicks 18 days of age were inoculated with leucotic organs from field cases of typical visceral lymphomatosis, as previously described,[5] 50 each with liver, spleen, heart, kidney, ovary, and blood, while 130 female chicks of the same lot were kept untreated as controls.

At the same time, 200 additional female chicks of the same lot, which had been previously treated with sex hormones, were inoculated with lymphomatous material from the same donors. In these chicks, however, a composite of material from all of the organs used as separate inoculums in the main transmission experiment was used, but the same amount, 3 cc., was given each bird intraperitoneally.

Administration of estrogens and androgens to these 200 female chicks had been initiated on Oct. 18, 1944. On Oct. 18, 21, 25, and 28, and on Nov. 1, 1944, 100 of these chicks had been given 0.2 mg. each subcutaneously of a-estradiol dipropionate (di-ovocylin), while, on the same dates, the remaining 100 chicks had been given 0.2 mg. each subcutaneously of testosterone propionate (perandren). Following the inoculation of these 200 chicks with the lymphomatous material on Nov. 3, 1944, 50 each from the estrogen-treated and the androgen-treated groups were given five additional doses of 0.2 mg. each of the same sex hormone they had received previously on Nov. 4, 8, 11, 15, and 18, 1944. Thus, 50 each received estrogen before, estrogen before and after, androgen before, and androgen before and after inoculation.

Ten weeks after inoculation, a series of monthly liver biopsies was obtained from 3 birds from each of the hormone-treated inoculated groups. These birds were operated on thereafter at monthly intervals for a period of ten months, or as long as the individual birds survived.

All birds in the hormone-treated groups which died during the course of the experiment were autopsied, and detailed autopsy records were made. After the termination of the experiment in November, 1945, all survivors were killed and examined. Tissues were saved

Published as journal paper No. 452 of the Purdue University Agricultural Experiment Station. These studies were supported in part by a grant from, and undertaken in cooperation with, the United States Regional Poultry Research Laboratory at East Lansing, Mich.

Sex hormones used in these experiments were supplied through the courtesy of Dr. F. E. Houghton of Ciba Pharmaceutical Products, Inc.

From the Department of Veterinary Science (Davis and Doyle), and the Department of Animal Husbandry (Andrews), Purdue University, Lafayette, Ind.

for microscopic study from all doubtful cases which died or were killed. All tissues were fixed in Bouin's fixative, embedded in paraffin, and sectioned. Duplicate slides of each tissue were stained one each in Harris's hematoxylin and phloxine, and one each for comparison in Harris's hematoxylin and orange G.

Results.—In the sex hormone-treated groups, the following results were obtained: Of the 50 chickens which received estrogen before inoculation with visceral lymphomatosis, 17 (34%) died of visceral lymphomatosis at an average age of 154.9 days (ave. 136.9 days after inoculation); of the 50 chickens which received estrogen both before and after inoculation, 17 (34%) died of the disease at an average age of 152 days (ave. 134 days after inoculation); of the 50 which received androgen before inoculation, 18 (36%) died of the disease at an average age of 186.1 days (ave. 168.1 days after inoculation); and of the 50 treated with androgen both before and after inoculation 22 (44%) died of visceral lymphomatosis at an average age of 181 days (ave. 163 days after inoculation). Thus, of the 100 estrogen-treated birds, 34 (34%) died of visceral lymphomatosis at an average age of 153.5 days (135.5 days after inoculation); of the 100 androgen-treated birds, 40 (40%) died of the disease at an average age of 183.3 days (ave. 165.3 days after inoculation); and of the 200 hormone-treated birds, 75 (37%) died of the disease at an average age of 169.6 days (151.6 days after inoculation). In evaluating these results, a high, early death loss in all four hormone-treated groups within the first month after inoculation, or before death from visceral lymphomatosis would ordinarily occur even in inoculated chickens, must be taken into consideration. These included 16 chicks of the group treated with estrogen before inoculation; 21 in the group treated with estrogen both before and after inoculation; 21 in the group treated with androgen before inoculation; and 10 in the group treated with androgen both before and after inoculation. The cause of death in these cases was pericarditis or peritonitis, or both, possibly caused by some contaminant accidentally introduced with the inoculum. While early visceral lymphomatosis could be detected by histologic study of the organs in some of these cases, it is quite possible that additional cases might have developed later, if the chickens had lived longer, which would have altered materially the percentages given above. Considering only the chicks which survived this early

TABLE I—Comparative Incidence of Visceral Lymphomatosis in Chickens Treated with Sex Hormones and Non-hormone-Treated Chickens

Group	No. in group	Cases of visceral lymphomatosis	Visceral lymphomatosis (%)	Average age at death (days) of cases	Days to death after inoculation of cases
Controls	130	22.0	16.2	242.0	
Visceral lymphomatosis-inoculated groups					
Liver	50	30.0	60.0	193.4	175.4
Kidney	50	17.0	34.0	211.4	193.4
Spleen	50	18.0	36.0	167.9	149.4
Blood	50	27.0	54.0	160.3	142.3
Heart	50	21.0	42.0	211.4	193.3
Ovary	50	16.0	32.0	201.0	183.0
Average		21.5	43.0	189.5	171.5
Hormone-treated and visceral lymphomatosis-inoculated groups					
Estrogen treated before inoculation	34	17	50.0	154.9	136.9
Estrogen treated before and after inoculation	29	17	57.9	152.0	134.0
All estrogen treated	63	34	53.9	153.5	135.5
Androgen treated before inoculation	29	18	62.0	186.1	168.1
Androgen treated before and after inoculation	40	22	55.0	181.0	163.0
All androgen treated	69	40	57.9	183.3	165.3
Total hormone treated	132	75	56.8	169.6	151.6

loss from other causes, the percentages of death from visceral lymphomatosis would be 50 per cent in the group of 34 which had received estrogen before inoculation; 57.9 per cent in the group of 29 which had received estrogen both before and after inoculation; 62 per cent in the group of 29 which had received androgen before inoculation; and 55 per cent in the group of 40 chicks which had received androgen both before and after inoculation. These data are compared with the results obtained in the main transmission experiment in table 1. Probably the average age at death in cases of visceral lymphomatosis in the various groups is significant. It was considerably lower in the estrogen-treated than in the androgen-treated groups and, in all hormone-treated groups, it was not only considerably lower than in controls but also slightly lower than in nonhormone-treated inoculated birds. (The data collected in the second sex hormone experiment described below, where the results were uncomplicated by any appreciable number of deaths from causes other than leucosis, should be more definitive.)

Meanwhile, only 9 cases of neurolymphomatosis appeared among all hormone-treated birds in this experiment. Two of these were in the group treated with estrogen before inoculation with visceral lymphomatosis, an incidence of 4 per cent in that group; 5 of the cases were in the group treated with androgen before inoculation, an incidence of 10 per cent; and 2 were in the group treated with androgen both before and after inoculation, an incidence of 4 per cent. Thus, the total incidence of neurolymphomatosis among all estrogen-treated birds was 2 per cent; among all androgen-treated birds, 7 per cent; and among all hormone-treated birds, 4.5 per cent; so that the incidence was no greater in any group than that of 10.8 per cent among the controls. Similarly, only 2 cases of granuloblastosis appeared among all the estrogen-treated birds (both were in the group receiving estrogen only before inoculation, making an incidence of 4% in that group), and 1 among all the androgen-treated birds (in the group receiving androgen only before inoculation), an incidence of 2 per cent among estrogen-treated birds, 1 per cent among androgen-treated birds, or 1.5 per cent among all hormone-treated birds. Thus, this form of leucosis was not increased in any group over the incidence of 5.4 per cent found in the controls. In addition, 1 case of mixed visceral lymphomatosis and granuloblastosis occurred in the group which received estrogen before inoculation. No cases of osteopetrosis or of erythroblastosis appeared in any hormone-treated group. Here again, the early death loss from intercurrent disease in all hormone-treated groups must be taken into consideration in an interpretation of these data.

The results of liver biopsy operations on chickens in this experiment closely paralleled those described in detail previously.[4]

Sex Hormone Experiment 2

A second and more complete study of the possible relationship of sex hormones to the incidence of visceral lymphomatosis was undertaken in November, 1945. Specifically, an attempt was made to obtain further data on the incidence of *both* spontaneous and transmitted avian visceral lymphomatosis in males, females, and capons, and the effect of the injection of estrogens and androgens on both spontaneous and transmitted visceral lymphomatosis in female chickens. A total of 1,100 chickens were used in the course of the experiment.

In the hope of speeding up transmission and increasing the percentage of takes, serial passage material was used as inoculum in the attempted transmission, instead of field material as heretofore in transmission work.

Successive liver biopsy operations on a representative group of chickens, and serial passage from the main transmission experiment were included in the study.

Experimental Procedure.—The following 14 groups of chicks were included in the main transmission experiment: 45 normal female controls; 45 normal male controls; 45 normal females inoculated with lymphomatous liver; 45 normal males inoculated with lymphomatous liver; 80 caponized male controls; 80 caponized males inoculated with lymphomatous liver; 45 females injected with five doses of estrogen;* 45 females injected with five doses of estrogen and inoculated with lymphomatous liver; 45 females injected with ten doses of estrogen; 45 females injected with ten doses of estrogen (5 before and 5 after inoculation) and inoculated with lymphomatous liver; 45 females injected with five doses of androgen;

*All doses of hormone consisted of 0.2 mg. given twice weekly.

45 females injected with five doses of androgen and inoculated with lymphomatous liver; 45 females injected with ten doses of androgen; and 45 females injected with ten doses of androgen (5 before and 5 after inoculation) and inoculated with lymphomatous liver.

The chicks used in the experiment were Barred Plymouth Rocks, hatched on Nov. 12, 1945. The chicks were sexed before they were used, but because of the percentage of error in sexing, a few males were included in the lots of hormone-injected females.**

On Nov. 14, 1945, when the chicks were 2 days old, the injection of estrogens and androgens was begun. On November 14, 17, 21, 24, and 28, 200 of the female chicks were each given 0.2 mg. of α-estradiol dipropionate subcutaneously, while on the same dates 200 other female chicks were given similar doses of testosterone propionate. After the inoculation of one half of each of these groups with lymphomatous material on November 29, treatment of one half of each of these groups was continued, so that one half of those which had previously received estrogen each were given additional 0.2-mg. doses of estrogen on Dec. 1, 5, 8, 12, and 15, 1945, while one half of those which had previously received androgen were given additional 0.2-mg. doses of androgen on the same dates.

Meanwhile, between Nov. 15 and 24, 1945, when the chicks were from 3 to 12 days of age, 400 male chicks of the same lot were caponized.

Before inoculation with lymphomatous material on November 29, the chickens of all lots were culled, leaving 45 chicks in each lot, excepting the caponized lots, in which 80 chicks each were included to allow for a possible high percentage of slips.

On Nov. 29, 1945, 350 chicks, including one half of the caponized chicks and one half of the hormone-treated chicks of each group, as well as normal males and normal females (in accordance with the plan outlined above) were inoculated with lymphomatous liver. The donor was a typical case of visceral lymphomatosis chosen from among the inoculated birds of serial passage 3 of the experiment of the previous year. (Thus, this entire transmission experiment represents a second serial passage 4 from transmission experiment 2, 1944 to 1945, and may be designated as serial passage 4 A in serial passage series of 1944 to 1945.) This bird showed marked enlargement of the liver and spleen, and microscopically showed marked lymphoblastic infiltration of the liver, spleen, heart, and kidneys. The liver was removed aseptically and triturated in sterile physiologic saline solution. The chicks were injected intraperitoneally with 3 cc. each of this material.

All chickens which died were autopsied. Samples of liver, spleen, heart, and kidney, as well as ovary in the case of females, of all doubtful cases were preserved for histologic study. All tissues saved were preserved in Bouin's fixative, embedded in paraffin, sectioned, and stained with Harris's hematoxylin and phloxine. Tissues from 87 chickens were preserved in this way and studied histologically.

On Jan. 22 to 25, 1946, a series of bimonthly liver biopsy operations was initiated on a group of 160 chicks, including 10 from each control and inoculated group, excepting the two caponized groups, and 20 from each of them. These operations were continued, at approximately eight-week intervals, on these 160 chicks throughout the course of the experiment, or until the deaths of the chickens. Six series of operations were performed during the year's experiment, or a total of 499 biopsy operations. Liver samples collected in this way were preserved in Bouin's fixative, embedded in paraffin, sectioned, and stained with Harris's hematoxylin and phloxine.

On April 18, 1946, 50 Barred Plymouth Rock chicks, 16 days old, were inoculated intraperitoneally with 3 cc. each of a trituration of lymphomatous liver in normal saline solution. The donor was a typical case of visceral lymphomatosis occurring among the caponized inoculated males of the main transmission experiment. Grossly, this bird showed markedly tumorous liver and kidneys and, microscopically, it showed marked lymphoblastic infiltration of the liver, spleen, heart, and kidneys. Fifty chicks of the same age and lot were kept untreated as controls for this serial passage group. The inoculated chicks of this group represented a fifth serial passage from the original field case used as a donor in transmission experiment 2, in 1944 to 1945, and, as the donor was taken from the main transmission experiment of transmission experiment 3 and thus represents serial passage 4 A of the 1944 to 1945 experiment, this serial passage group may be designated serial passage 5 A.

Between Dec. 13, 1946, and Jan. 24, 1947, all survivors, both controls and inoculated birds, from the main transmission experiment were killed and examined. On Jan. 24, 1947, all survivors of the serial passage groups were killed and examined.

RESULTS

Results of Transmission.—Of the 342 birds inoculated with lymphomatotic liver which eventually came to autopsy before the end of the experiment, 231 (67.5%) developed visceral lymphomatosis. Of the 323 control birds which eventually came to

**These were eliminated from the data concerning the incidence of visceral lymphomatosis in summarizing it, but are included in the data concerning other forms of leucosis.

autopsy, 60 (18.6%) developed the disease. Thus, the incidence was increased from a spontaneous incidence of 18.6 to 67.5 per cent, or more than three times as much in inoculated birds.

During the same period, 14 cases of neurolymphomatosis (4.1%) appeared among the 342 inoculated birds, as compared with 23 cases (7.1%) among the 323 control birds. Three cases of granuloblastosis (0.9%) occurred among the inoculated birds, as compared with 4 cases (1.2%) among the controls. One bird among the caponized controls, a slip, showed both granuloblastosis and iritis, and thus has been included both among the birds showing neurolymphomatosis and among the birds showing granuloblastosis, and the corresponsing percentages are so figured. No cases of osteopetrosis or erythroblastosis appeared in either the inoculated or control groups.

Thus, in this third transmission experiment, as in the previous two (transmission experiment 1, 1943 to 1944, and transmission experiment 2, 1944 to 1945[3, 5]), no type of avian leucosis other than visceral lymphomatosis was increased in the inoculated birds as compared with the control birds, suggesting that visceral lymphomatosis was again transmitted (for the third time) as a separate entity distinct from other lymphomatous forms of leucosis (neurolymphomatosis and osteopetrosis) and from the myelogenous forms of leucosis (erythroblastosis and granuloblastosis).

The average age at death of visceral lymphomatosis among inoculated birds was 163.5 days (146.5 days after inoculation), as compared with 235 days of age at death among spontaneous cases of visceral lymphomatosis among control birds. In cases of neurolymphomatosis among the inoculated birds, the average age at death was 164.8 days (157.8 days after inoculation) as compared with an average age at death of 206.5 days of cases of neurolymphomatosis among control birds. In cases of granuloblastosis among inoculated birds, the average age at death was 131 days (114 days after inoculation) as compared with an average age at death of 273 days among control cases of granuloblastosis. The mixed case showing both neurolymphomatosis and granuloblastosis died at 420 days of age. Thus, the average age at death of cases of all types of avian leucosis was considerably lower among inoculated birds than among control birds. In the case of visceral lymphomatosis, the average difference (71.5 days) is highly significant. However, in the case of granuloblastosis, and possibly also in the case of neurolymphomatosis, the number of cases is so small in both inoculated and control groups that these averages have no statistical significance.

Results Involving Sex Hormone Relationships.—In a comparison of the incidence of both spontaneous and transmitted visceral lymphomatosis in females, males, capons, and slips, the following results were obtained: Of 47 inoculated normal females, 32 (68%) showed visceral lymphomatosis, as compared with 12 cases (30%) among 40 control females; of 43 inoculated normal males, 27 (62.8%) showed visceral lymphomatosis, as compared with 4 cases (9.1%) among 44 control males; of 65 inoculated capons, 42 (64.6%) showed visceral lymphomatosis, as compared with 7 cases (14%) among 50 control capons; while of 13 inoculated slips (a bird was considered a slip if autopsy showed any regeneration of the testis), 9 (69.2%) showed visceral lymphomatosis, as compared with 2 cases (8.7%) among 23 control slips. If capons and slips are considered together, 51 cases of visceral lymphomatosis occurred among 78 inoculated birds (65.4%), while control capons and slips together showed 9 cases among 73 controls (12.3%). If slips and normal males are considered together, 56 inoculated birds showed 36 cases of visceral lymphomatosis (64.3%), while 6 cases (8.9%) appeared among 67 control slips and normal males. Thus, in inoculated birds, the incidence of visceral lymphomatosis was 69.2 per cent in slips, 68.0 per cent in normal females, 65.4 per cent in capons and slips combined, 64.6 per cent in capons alone, 64.3 per cent in males and slips combined, and 62.8 per cent in normal males. In controls, the spontaneous incidence of visceral lymphomatosis was 30 per cent in females, 14 per cent in capons, 12.3 per cent in capons and slips combined, 9.1 per cent in normal males, 8.9 per cent in normal males and slips combined, and 8.7 per cent in slips alone.

In the hormone-treated groups, the following results were obtained: Of the 38 females which received estrogen before inoculation with visceral lymphomatosis, 26 (68.4%) died of visceral lymphomatosis at

an average age of 168.8 days (151.8 days after inoculation); of the 41 females which received estrogen both before and after inoculation, 27 (65.8%) died of visceral lymphomatosis at an average age of 163.3 days (ave. 146.3 days after inoculation); of the 39 females which received androgen before inoculation with visceral lymphomatosis, 28 (71.8%) died of visceral lymphomatosis at an average age of 167.8 days (150.8 days after inoculation); and of the 38 females which received androgen both before and after inoculation, 24 (63.1%) died of visceral lymphomatosis at an average age of 140.0 days (123.0 days after inoculation). Thus, of all estrogen-treated and inoculated females, 67 per cent died of visceral lymphomatosis at an average age of 165.9 days (ave. 148.9 days after inoculation); while, of all androgen-treated and inoculated females, 67.5 per cent died of visceral lymphomatosis at an average age of 155.0 days (ave. 138.0 days after inoculation).

Among the hormone-treated controls, the following results were obtained: Of the 36 females which received five doses of estrogen, 8 (22.2%) died of visceral lymphomatosis at an average age of 178 days; of the 37 females which received ten doses of estrogen, 8 (21.6%) died of visceral lymphomatosis at an average age of 228.5 days; of the 35 females which received five doses of androgen, 4 (11.4%) died of visceral lymphomatosis at an average of 267.0 days of age; and of the 36 females which received ten doses of androgen, 11 (21.9%) died of the disease at an average age of 205.2 days. Thus, of all 73 estrogen-treated control birds, 21.9 per cent died of visceral lymphomatosis at an average age of 205.2 days; while, of the 71 androgen-treated control birds, 19.5 per cent died of the disease at an average age of 281.4 days. These results are summarized in table 2.

Meanwhile, several cases of other forms of avian leucosis appeared among the hormone-treated groups. A total of 6 cases of neurolymphomatosis occurred among the hormone-treated inoculated groups, which were distributed as follows: One case (2.4%) of neurolymphomatosis in the group given estrogen only before inoculation died at 74 days of age; 2 cases (4.4%) among the birds given estrogen both before and after inoculation died at an average age of 232.5 days; 1 case (2.3%) among the birds treated with androgen only before inoculation died at 119 days of age; and 2 cases (4.6%) treated with androgen both before and after inoculation died at an average age of 99 days. Two cases of myelogenous leucosis, both granuloblastic, appeared among the hormone-treated and inoculated groups. One of these (2.2%) occurred in the group of birds receiving estrogen both before and after inoculation with visceral lymphomatosis and died at 141 days of age; the other (2.3%) occurred in the group which received androgen both before and after inoculation with visceral lymphomatosis and died at 214 days of age. A total of 8 cases of neurolymphomatosis appeared among the hormone-treated control birds, which were distributed as follows: Two cases (4.9%) appeared in the group receiving five doses of estrogen, and these died at an average age of 159 days; 2 cases (5.0%) appeared among the birds which received ten doses of estrogen, and these died at an average age of 185 days; and 4 cases (9.3%) occurred in the group which received five doses of androgen, and these died at an average age of 174.5 days. Three cases of myelogenous leucosis, all granuloblastic in type, appeared in the hormone-treated control groups as follows: One case (2.3%) occurred in the group which received five doses of estrogen and died at 150 days of age; and 2 cases (4.8%) in the group which received androgen both before and after inoculation died at an average age of 261 days. Thus, among the hormone-treated birds, as among the other groups in the transmission experiment, no type of leucosis other than the visceral lymphomatosis which was inoculated was increased in the inoculated groups as compared with the control group.

Results of Liver Biopsy Operations.—Histologic findings in the liver biopsy studies undertaken as a part of this experiment are closely similar to those obtained in previously reported experiments.[4, 5]

Results of Serial Passage.—In the serial passage groups, in the 45 inoculated chickens which could be accounted for at the end of the experiment, 20 (44.4%) died of visceral lymphomatosis at an average age of 202.3 days, (186.3 days after inoculation); and 4 (8.8%) died of neurolymphomatosis at an average age of 192 days (176 days after inoculation with visceral lymphomatosis). Of the 45 controls, 3 (6.6%) died

at an average age of 243 days, while none died of neurolymphomatosis; and 2 (4.4%) died of granuloblastosis at an average age of 238.5 days. Thus, the incidence of visceral lymphomatosis was increased from 6.6 per cent in the control group to 44.4 per cent in the inoculated group, or more than six times as much in the inoculated group. The 8.8 per cent of neurolymphomatosis found in the inoculated group as compared with none in the control group is well within the average incidence which might be expected to occur spontaneously in comparable groups of chickens, and probably does not represent an increase resulting from the inoculation of lymphomatous material from a case of visceral lymphomatosis.

As compared with the results of the main transmission experiment, these results show a markedly greater increase in this serial passage than in the previous one which the main transmission experiment represents.

Histologic Results.—Tissues from 99 chickens, both inoculated and control birds, were prepared for microscopic study and examined. Of these, 87 were from the main transmission experiment, and 12 were from the serial passage groups. Fifty-eight of these birds, including 54 from the main experiment and 4 from the serial passage groups, were found to show visceral lymphomatosis; 8, including 7 from the main experiment and 1 from the serial passage groups, showed granuloblastosis; 2, both from the main experiment, showed neurolymphomatosis; and 31, including 24 from the main experiment and 7 from the serial passage groups, were negative for all forms of leucosis.

Of the 48 positive cases of visceral lymphomatosis from which liver tissues were studied, 43 (89.5%) showed lesions in the liver; of the 45 hearts from positive cases which were examined, 38 (84.3%) showed lymphomatous lesions; of the 44 kidneys from positive cases which were examined,

TABLE 2—Incidence of Spontaneous and Transmitted Visceral Lymphomatosis in Sex Hormone-Treated and Non-hormone-Treated Groups of Chickens

Sex	Treatment	No. of birds	Percentage developing visceral lymphomatosis	Average age at death of cases	Days to death after inoculation of cases
		Control group			
Females	Control	40	30.0	220	
Males	Control	44	9.1	223	
Capons[1]	Control	50	14.0	224	
Slips[1]	Control	23	8.7	204	
		Inoculated[2] group			
Females	Inoculated	47	68.0	171.4	154.4
Males	Inoculated	43	62.8	158.5	141.5
Capons[1]	Inoculated	65	84.6	153.7	135.7
Slips[1]	Inoculated	13	69.2	200.7	183.7
		Hormone-treated[3] control group			
Females	5 doses of estrogen[4]	36	27.2	178.0	
Females	10 doses of estrogen[5]	37	21.6	228.5	
Females	All estrogen treated	73	21.9	205.2	
Females	5 doses of androgen[4]	35	11.4	267.0	
Females	10 doses of androgen[5]	36	30.6	286.6	
Females	All androgen treated	71	19.5	281.4	
		Hormone-treated[3] inoculated[2] group			
Females	5 doses of estrogen[4] before inoculation	38	68.4	168.8	151.7
Females	10 doses of estrogen[5] 5 before, 5 after inoculation	41	65.8	163.3	146.3
Females	All estrogen treated	79	67.0	165.9	148.9
Females	5 doses of androgen[4] before inoculation	39	71.8	167.8	150.8
Females	10 doses of androgen[5] 5 before, 5 after inoculation	38	63.1	140.0	123.0
Females	All androgen treated	77	67.5	155.0	138.0

[1]Caponized between 3 and 12 days of age.
[2]Inoculated at 17 days of age with 3 cc. each intraperitoneally of a trituration of lymphomatous liver in sterile saline solution.
[3]Sex hormones used were a-estradiol dipropionate and testosterone propionate (Ciba), administered subcutaneously in doses of 0.2 mg. each in sesame oil.
[4]Doses were given at 2, 5, 9, 12 and 16 days of age.
[5]Doses were given at 2, 5, 9, 12, 16, 19, 23, 26, 30, and 33 days of age.

34 (77.3%) showed lymphomatous lesions; of the 45 positive spleens examined, 34 (75.5%) showed lymphomatous lesions; and of the 15 ovaries from positive cases which were examined, 11 (73.3%) showed lymphomatosis; and the tumorous vas deferens from 1 positive case which was examined microscopically was lymphomatous. Of the cases of granuloblastosis studied histologically, seven of the seven livers examined and two of the two ovaries examined (100% in each case) showed granuloblastic lesions; as did four of the five kidneys (80%); three of the four hearts (75%); and three of the five spleens (60%).

SUMMARY AND CONCLUSIONS EXPERIMENT 1

1) The incidence of visceral lymphomatosis was 16.2 per cent in untreated controls, and 43 per cent in all groups inoculated with visceral lymphomatosis but not treated with sex hormones. In groups inoculated with visceral lymphomatosis and variously treated with sex hormones, the incidence of visceral lymphomatosis was 50 per cent in groups of chickens treated with estrogen before inoculation and 57.9 per cent in chickens treated with estrogen both before and after inoculation, 62 per cent in the group treated with androgen before inoculation, and 55 per cent in the group treated with androgen both before and after inoculation. However, because of high early death losses from other causes in all these groups, the significance of these percentages is in doubt.

2) The incubation period for visceral lymphomatosis in the various hormone-treated groups was apparently shortened from the average age at death of 242 days among control cases, and the average age at death of 189.5 days of inoculated cases which were not hormone treated, to an average of 154.9 days at death in cases treated with estrogen before inoculation, 152 days in cases treated with estrogen both before and after inoculation, 186.1 days in cases treated with androgen before inoculation, and 181 days in cases treated with androgen both before and after inoculation. Thus, cases in all hormone-treated groups died sooner than the nonhormone-treated cases. The difference between the androgen-treated and the nonhormone treated groups is negligible, but the cases in estrogen-treated groups died considerably sooner on the average than those in either androgen-treated or nonhormone-treated groups.

SUMMARY AND CONCLUSIONS EXPERIMENT 2

1) The incidence of visceral lymphomatosis was increased by inoculation with lymphomatous liver from an average incidence of 18.5 per cent in the control groups to an average incidence of 67.5 per cent, or more than three times as much in the inoculated groups. The inoculating tissue was taken from a serial passage case of visceral lymphomatosis from the previous transmission experiment.

2) Visceral lymphomatosis was transmitted for the third time, apparently as a separate entity distinct from all other forms of avian leucosis, including neurolymphomatosis.

3) The incubation period for visceral lymphomatosis was apparently shortened by inoculation with lymphomatous liver from an average of 235 days at death in cases of visceral lymphomatosis from all control groups to an average of 163.5 days at death in cases of visceral lymphomatosis in all inoculated groups. The average age at death from neurolymphomatosis and granuloblastosis was also lower in inoculated cases than in control cases, suggesting that inoculation of visceral lymphomatosis generally lowered the resistance of the chickens inoculated even when clinical cases of visceral lymphomatosis did not develop.

4) Among inoculated birds, the incidence of visceral lymphomatosis in females was 68.0 per cent; in normal males, 62.8 per cent; in capons, 64.6 per cent; in slips, 69.2 per cent; in capons and slips together, 65.4 per cent; and in males and slips together, 64.3 per cent. Among control birds, the spontaneous incidence of visceral lymphomatosis in females was 30 per cent; in males, 9.1 per cent; in capons, 14 per cent; in capons and slips together, 12.3 per cent; and in males and slips, 8.9 per cent. Thus, in both inoculated and control birds, the incidence of visceral lymphomatosis was higher in females and in capons than in normal males.

5) In the hormone-treated inoculated groups, 68.4 per cent of the females which received estrogen before inoculation with lymphomatous liver, 65.8 per cent of the females which received estrogen both before and after inoculation, 71.8 per cent of

the females which received androgen before inoculation, and 63.1 per cent of the females which received androgen both before and after inoculation died of visceral lymphomatosis. In the hormone-treated control groups, 22.2 per cent of the females which received the smaller amount of estrogen, 21.6 per cent of the females which received the larger amount of estrogen, 11.4 per cent of the females which received the smaller amount of androgen, and 30.6 per cent of the females which received the larger amount of androgen died of visceral lymphomatosis.

6) Of birds which died of visceral lymphomatosis, those in all inoculated groups, including all hormone-treated groups, died at an earlier average age than cases in comparable control groups.

7) Differences between inoculated groups in the incidence of visceral lymphomatosis are slight, possibly because the use of serial passage material as inoculum produced an over-all incidence so high as to obscure differences which might have been more apparent with the use of less virulent material. Between control groups, the differences are greater, but the unusually high incidence of visceral lymphomatosis among normal females (as compared with spontaneous incidence, and the differences between males and females in previous experiments) makes the interpretation of the results of hormone treatment difficult. Apparently, both caponization and treatment with sex hormones, regardless of whether estrogens or androgens were used, increased, at least slightly, susceptibility of chickens to visceral lymphomatosis. Such increased susceptibility would appear to be a matter of hormonal imbalance rather than a specific effect of a lack or excess of either estrogen or androgen.

8) In the serial passage groups, 44.4 per cent of inoculated birds died of visceral lymphomatosis at an average age of 192 days, while 6.6 per cent of the controls died of the disease at an average age of 243 days, or the incidence in the inoculated birds was more than six times as great as in the controls. As compared with the previous serial passage (in this case, the main transmission experiment), this serial passage shows a much greater incidence than the previous one, or an increase of 600 per cent as compared with an increase of 300 per cent. The average age at death of cases of visceral lymphomatosis in this serial passage, however, was slightly higher than that of cases in the previous serial passage.

9) Of all tissues from cases of visceral lymphomatosis which were examined histologically, 89.5 per cent of the livers, 84.3 per cent of the hearts, 77.3 per cent of the kidneys, 75.5 per cent of the spleens, and 73.3 per cent of the ovaries showed lymphomatous lesions.

References

[1]Burmester, B. R.: The Incidence of Lymphomatosis Among Male and Female Chickens. Poult. Sci., 24, (1945):469-472.

[2]Burmester, B. R., and Nelson, N. M.: The Effect of Castration and Sex Hormones upon the Incidence of Lymphomatosis in Chickens. Poult. Sci., 24, (1945):509-515.

[3]Davis, O. S., and Doyle, L. P.: Studies in Avian Leucosis. I. The Transmissibility of Visceral Lymphomatosis. Am. J. Vet. Res., 8, (1947):103-112.

[4]Davis, O. S., and Doyle, L. P.: Studies in Avian Leucosis. II. The Use of Biopsy Technique in the Study of Visceral Lymphomatosis. Am. J. Vet. Res., 8, (1947):113-119.

[5]Davis, O. S., and Doyle, L. P.: Studies in Avian Leucosis. IV. Further Transmission of Visceral Lymphomatosis. Am. J. Vet. Res., 10, (1949):85-91.

[6]Gardner, W. U., and Dougherty, T. F.: The Leucogenic Action of Estrogens in Hybrid Mice. Yale J. Biol. & Med., 17, (1944):75-90.

[7]Gardner, W. U., Dougherty, T. F., and Williams, W. L.: Lymphoid Tumors in Mice Receiving Steroid Hormones. Cancer Res., 4, (1944):73-87 (9 fig.).

[8]Gardner, W. U., Kirschbaum, A., and Strong, L. C.: Lymphoid Tumors in Mice Receiving Estrogens. Arch. Path., 29, (1940):1-7.

[9]Marine, D., and Rosen, S. H.: Increase in the Incidence of Lymphomatosis in Male Fowls by Castration. Am. J. Cancer, 39, (1940):315-318.

[10]Marine, D., and Rosen, S. H.: Sex Hormones and Lymphomatosis in Fowls. Proc. Soc. Exptl. Biol. and Med., 47, (1941):61.

[11]Silberberg, M., and Silberberg, R.: Significance of the Age Factor and Sex Glands in Experimental Leucemia of Mice. Proc. Soc. Exptl. Biol. and Med., 58, (1945):347-348.

Reinfection of Chickens Vaccinated by the Intranasal Method with Live B1 Newcastle Disease Virus

E. R. DOLL, M.A., D.V.M., M. ELIZABETH WALLACE, B.S., M.S., and WILLIAM H. McCOLLUM, B.S., M.S.

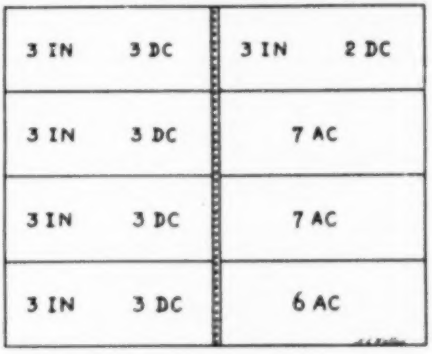
Lexington, Kentucky

THE B1 STRAIN of Newcastle disease virus (NDV) was introduced by Hitchner and Johnson[1] and was reported to be of such sufficiently low virulence that it could be employed by intranasal instillation (IN) for immunization of day-old chicks hatched from susceptible hens. In the course of our studies toward determining the nature and quality of immunity obtained from the B1 strains of NDV, it was observed that the hemagglutination-inhibition (HI) response resulting from intranasal inoculation of chickens with this virus was of short duration.[2,3] Also, chickens that had lost most or all of their demonstrable HI antibody often showed mild symptoms of respiratory infection following intranasal or intratracheal challenge with virulent NDV. The same chickens, when subjected to periodic HI testing, showed a characteristic anamnestic response,[2,3,4] a reaction observed by Brandly *et al.*[5] and Hanson *et al.*[6] This investigation was undertaken to obtain information on the nature of the anamnestic response and to determine if it resulted from an active reinfection with NDV. Also, information was sought for characterization of the apparent reinfection and the nature and quality of immunity resulting from intranasal vaccination with the B1 strain of NDV.

MATERIALS AND METHODS

The chickens used in this study, standard-bred New Hampshire Reds, were hatched from susceptible hens. They were reared in electrically heated brooders and fed a commercial baby chick starting mash. When 3 weeks old, the chicks were inoculated intranasally with 0.05 cc. of B1 NDV, undiluted amnioallantoic fluid from the seventy-sixth embryo passage. When they were 5 weeks old, the chickens were transferred to growing batteries and were maintained in isolation until they were 9 weeks old. At this age, one group of 19 chickens was injected intramuscularly with 0.1 cc. of formalin-killed GB* strain of NDV, and a second group of 19 chickens received 0.1 cc. of formalin-killed GB strain of NDV intranasally. Before inactivation, the virus had an embryo infectivity titer of 10^9 lethal dose$_{50}$ (l.d.$_{50}$) per 0.05 cc. For the live virus challenge at 11 weeks of age, a group of 15 chickens was exposed by IN with 10^6 embryo l.d.$_{50}$ of the GB virus. Another group of 14 chickens was exposed by direct contact (DC) with those inoculated intranasally, and a third group of 20 chickens was subjected to airborne contact (AC) exposure by placing them in separate sections of the growing battery. The dispersal and identification of the chickens employed for the live virus challenge are shown in fig. 1.

Fig. 1—Dispersal in growing battery of chickens employed for reinfection experiment.



IN=10^6 embryo l.d.$_{50}$ GB virus intranasally.
DC=direct contact exposure.
AC=airborne contact exposure.

The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the director.

This study was conducted as a segment of Research and Marketing Act Regional Project S6—Diagnosis and Control of Newcastle Disease of Poultry. The cooperating agencies under this project are Kentucky, Virginia, and Texas Agricultural Experiment Stations. The research reported in this paper was done solely at the Kentucky station.

From the Department of Animal Pathology, University of Kentucky, Lexington.

*GB is the designation for a strain of NDV isolated by Dr. W. A. Boney, Jr., School of Veterinary Medicine, Texas A. & M. College, College Station.

Blood samples for preëxposure HI titers were obtained three days before administration of the killed NDV and two weeks before reëxposure to the living NDV. Following reëxposure, blood samples for HI tests were obtained on the fourth, fifth, sixth, seventh, eighth, and tenth days after inoculation.

The HI test employed twofold serial dilutions of fresh serum in 0.25-cc. quantity, 10 hemagglutination units of NDV strain Ky-127 contained in 0.25 cc. of physiologic saline, and 0.5 cc. of 0.5 per cent chicken red cells. The serum-virus mixture was incubated ten minutes at room temperature. Agglutination patterns were read at forty-five minutes, using complete inhibition of agglutination as the end point.

Isolations of NDV from the chickens subjected to live virus challenge exposure were made from tracheal fluids obtained with cotton swabs on 10-in. lengths of 16-gauge aluminum wire. The swabs were inserted into the trachea to a point near the bifurcation of the bronchi and removed slowly while rotating the wire between the index finger and thumb. A separate swab was used for each chicken. The swabs from each group of chickens were washed in 25 cc. of saline containing 5,000 units per cubic centimeter each of penicillin and streptomycin. This mixture was allowed to stand at room temperature for two hours before inoculation into the allantoic cavity of embryonated chicken eggs, using 0.5 cc. of inoculum for each embryo. The ages of the embryos were 10, 11, and 12 days for swabs made on three successive days.

IMMUNIZATION AND CHALLENGE EXPOSURE

Following intranasal inoculation with the B1 strain of NDV at 3 weeks of age, all of the chickens developed significant HI antibody with titers ranging from 1 : 80 to 1 : 640, indicating that active infection and an immunizing reaction had occurred. Six weeks later, the HI titers of the chickens varied from less than 1 : 10 to 1 : 80. Average HI titers of the prechallenge samples from the different groups of chickens are shown in table 1.

For the group of 19 chickens receiving 0.1 cc. of formalin-killed GB strain of NDV intranasally, there was a slight increase in average HI titer throughout the bleeding period, beginning on the fourth day and ending on the tenth day after inoculation. This increase in average titer did not exceed a twofold difference, which is within the limits of variation for the testing procedure. The group of 19 chickens receiving 0.1 cc. of formalin-killed GB strain of NDV intramuscularly showed a gradual increase in average HI titer throughout the bleeding period. The average titer at ten days following injection of the killed virus was sixfold higher than that obtained for the prechallenge samples.

The average HI titer of 15 chickens receiving intranasal inoculation of 10^5 embryo $l.d._{50}$ of living GB strain of NDV was lower at four days following exposure than for the prechallenge bleeding. In this group, ascension of HI titers began on the fifth day and showed a rapid increase typical of the anamnestic response on the sixth and seventh days. Significant increase in the average HI titer of 14 chickens, group DC, exposed by direct contact with those inoculated intranasally, occurred on the sixth day. This was followed by a rapid increase in the average HI titer on the seventh and eighth days. Twenty chickens, group AC, exposed in separate compartments of the battery did not show an increase in average titer until the seventh day, and the average titer was still rising at the tenth day following exposure. The HI response of the different groups is summarized in table 1.

At ten days after exposure, all of the 15 chickens inoculated intranasally with live virus had HI titers of 1 : 1,280, the highest serum dilution tested. Thirteen of the chickens exposed by direct contact had HI titers of 1 : 1,280, 1 had a titer of 1 : 20, and 1 failed to show an increase of HI titer.

Of the 20 chickens exposed in separate compartments of the battery, with the first cases probably being acquired by airborne transmission, only 11 showed significant elevation of their HI titers at ten days following expo-

TABLE 1—Hemagglutination-Inhibition Response Resulting from Challenge Exposure with GB Virus at Nine Weeks of Age, Six Weeks Following Intranasal Vaccination with B1 Virus at Three Weeks of Age

Route of exposure	No. of chickens	Prechallenge HI titer (ave.)	Group average HI titers obtained at specified days following exposure to GB virus					
			4	5	6	7	8	10
IN	15	16*	6	20	161	613	800	1280
DC	14	10*	3	11	28	222	504	1190
AC	20	6*	2.5	5	6	45	224	508
KVIM	19	6†	6	11	17	20	25	36
KVIN	19	15†	17	18	20	21	27	28

IN = intranasal; DC = direct contact; AC = airborne contact; KVIM = killed virus intramuscularly; KVIN = killed virus intranasally; * = two weeks before live virus exposure; † = three days before killed virus exposure.

sure. At thirteen days, however, 16 chickens had HI titers of 1 : 640 or 1 : 1,280 and 4 chickens showed no inhibition of agglutination at the 1 : 10 serum dilution.

On the third day following exposure, tracheal swabs were made on the IN group. This material was prepared for chicken embryo inoculation as outlined above. Newcastle disease virus was obtained from the swabs in sufficient quantity to cause death of 30, 10-day-old embryos within thirty-six hours. The presence of NDV in the allantoic fluid from each embryo was confirmed by the hemagglutination test. On the fourth day following exposure, tracheal swabs were made on the DC and AC groups. Inoculum for each group prepared from these swabs was injected into 30, 11-day embryos and caused death of all embryos within forty hours. The hemagglutination test showed NDV to be present in the allantoic fluid of all embryos. Tracheal swabs made from the AC group on the fifth day following exposure gave similar results, causing death of 30, 12-day embryos within forty hours.

DISCUSSION

The results obtained in this study indicate that the anamnestic response resulting from intranasal challenge exposure of chickens previously vaccinated intranasally with the B1 strain of NDV may be regarded as evidence of active reinfection. Vaccinated chickens receiving IN instillation of killed NDV showed no rise in HI titers. Vaccinated chickens receiving intramuscular injection of killed NDV showed only a slight increase in HI titer, as compared to the response obtained in similar chickens following three methods of exposure to living NDV. The increase in HI titers appeared sooner in the IN group than in the DC and AC groups. This variation may have resulted from difference in the amount of NDV available to initiate the infection. Also, it is probable that the chickens of the DC and AC groups acquired infection at succeeding periods of time after the intranasal challenge inoculations were made. This was evident by failure to obtain serologic evidence of reinfection of 9 chickens in the AC group ten days after inoculation of the IN group. The increase of HI titers in the DC and AC groups indicated that the amount of virus available by direct contact and airborne contact exposure was sufficient to initiate reinfection of the chickens previously vaccinated with the B1 virus. Likewise, mild and transient symptoms of respiratory infection occurred in some chickens in each of the three groups. Isolation of NDV from tracheal swabs made on chickens of the DC group on the fourth day after the first possible exposure, and from the AC group on the fourth and fifth days, also provided evidence that the challenge virus had become established in the respiratory tract of the chickens.

Approximately equal quantities of serum, from the blood samples obtained for the prechallenge HI tests, were pooled for serum-neutralization tests. The pooled serum had a NDV-neutralizing capacity of 10^4 embryo $l.d._{50}$ per 0.05 cc. when tested against NDV strain Ky-201. Under the conditions of this experiment, in which significant virus-neutralizing antibody was present in the chickens subjected to challenge exposure, the infectious process was reduced in severity to such an extent that systemic illness and fatal infection did not occur in any of the chickens. The efficiency of this protection was shown by the fatal infection of 34 of 35 susceptible chickens, 7 weeks old, receiving intranasal inoculation of 0.05 cc. of the same embryo passage of the GB strain of NDV. The susceptible chickens were four weeks younger than the B1 vaccinated group, at the time of exposure. An age factor of four weeks, however, does not adequately explain the difference in susceptibility.

In explanation of the resistance to fatal or systemic infection of the B1-vaccinated chickens, it appears that the circulating antibody was effective in limiting the extent of the reinfection. The capacity of the GB strain of NDV to cause generalized paralysis of nearly all of the infected susceptible chickens[3] further emphasizes the limited extent of the reinfection. If the initial point of attack by NDV is the respiratory mucosa, the presence of only mild respiratory symptoms and the isolation of NDV from tracheal swabs suggest that the reinfection was limited to the respiratory tract. In this event, it may be reasoned that the epithelial surfaces of the respiratory tract were in a susceptible condition which permitted establishment and growth of NDV. Further invasion by the NDV, however, appeared to have been prevented by the circulating antibody and systemic infection did not occur. For the exanthematous diseases and influenza of man, a similar mechanism of immunity and reinfection was presented by Francis.[7]

If the anamnestic response resulting from challenge exposure in a group of chickens vaccinated by intranasal inoculation with the B1 strain of NDV may be regarded as evidence of reinfection, unpublished data[8] indicate that chickens vaccinated intranasally at 1 day of age have been reinfected forty days later with NDV strain Ky-50, and chickens vaccinated intranasally at 3 weeks of age have been reinfected seven weeks later with the B1 strain of NDV, but chickens vaccinated at 5 weeks of age were not reinfected four weeks later with NDV strain Ky-50. Chickens vaccinated intramuscularly with the B1 virus at 5 weeks of age showed serologic evidence of reinfection with virus Ky-50 eighteen days later. Intratracheal challenge was employed in all of these groups.

It appears that the reinfection occurring in chickens immunized with the B1 strain of NDV may not be a problem in regard to mortality and systemic illness of the affected chickens. Except for very mild symptoms of respiratory infection, infrequent sneezing or coughing in some chickens, the reinfection has been subclinical. Chickens challenged with virulent virus at nine to eleven weeks following intranasal vaccination under 1 week of age have, with very few exceptions, shown no effect on activity or feed consumption as a result of the reinfection.[2] Hitchner and Johnson,[1] and Hitchner[9] have obtained similar results from challenge exposure for periods as long as one year following vaccination of day-old chickens.

SUMMARY

Chickens hatched from susceptible hens and vaccinated at 3 weeks of age by intranasal inoculation with the B1 strain of NDV were reinfected with the GB strain of NDV at fifty-one days following vaccination. Reinfection was accomplished by intranasal (IN), direct contact (DC), and airborne contact (AC) exposure.

The anamnestic response of hemagglutination-inhibition (HI) titers following intranasal challenge exposure of B1-vaccinated chickens which have lost the demonstrable HI antibody resulting from vaccination is indicative of active reinfection by NDV. The reinfection was subclinical in nature and appeared to be limited to the respiratory tract. The reinfection was not serious as a cause of morbidity and mortality, but may have implications in the epizoötiology of Newcastle disease.

References

[1]Hitchner, S. B., and Johnson, E. P.: A Virus of Low Virulence for Immunizing Fowls Against Newcastle Disease. Vet. Med., *43*, (1948) :525-530.

[2]Doll, E. R., McCollum, W. H., and Wallace, M. E.: Immunization Against Newcastle Disease with a Virus of Low Virulence. Vet. Med., *45*, (1950): 231-236.

[3]Doll, E. R., McCollum, W. H., and Wallace, M. E.: Immunization of Chicks Hatched from Hens Immunized Against Newcastle Disease. Vet. Med. In press.

[4]Doll, E. R., Wallace, M. E., and McCollum, W. H.: Interpretation of Serologic Procedures for Diagnosis of Newcastle Disease. Am. J. Vet. Res., *11*, (1950): 265-271.

[5]Brandly, C. A., Hanson, R. P., Lewis, S. H., Winslow, N. S., Hoyt, H. H., Pritchard, W. R., and Nerlinger, C. M.: Variables and Correlations in Laboratory Procedures for Newcastle Disease Diagnosis. Cornell Vet., *37*, (1947) :324-336.

[6]Hanson, R. P., Brandly, C. A., and Winslow, N. S.: Divergence of Serum Neutralization and Hemagglutination Inhibition Activity of Newcastle Immune Sera. Proc. Forty-eighth Meet. Am. Soc. Bacteriologists, *1*, (1948) :35.

[7]Francis, Thomas, Jr.: Mechanisms of Infection and Immunity in Virus Diseases of Man. Bact. Rev., *11*, (1947) :147-156.

[8]Doll, E. R.: Unpublished data.

[9]Hitchner, S. B.: Further Observations on a Virus of Low Virulence for Immunizing Fowls Against Newcastle Disease (Avian Pneumoencephalitis). Cornell Vet., *40*, (1950) :60-70.

COPYRIGHT 1950 BY AMERICAN VETERINARY MEDICAL ASSOCIATION

Printed in the U.S.A.

for example

...in cancer case study

The high-pressure mercury arc of the Scopicon is the brightest light source ever employed for microprojection. The focal spot employed is roughly 1 mm. square: its pinpoint character permits flickerless projection of crisply detailed images up to ten feet across *even under oil-immersed microscope objectives.* The white color exhibits the various biological stains to splendid advantage May we send you the brochure describing this versatile new instrument?

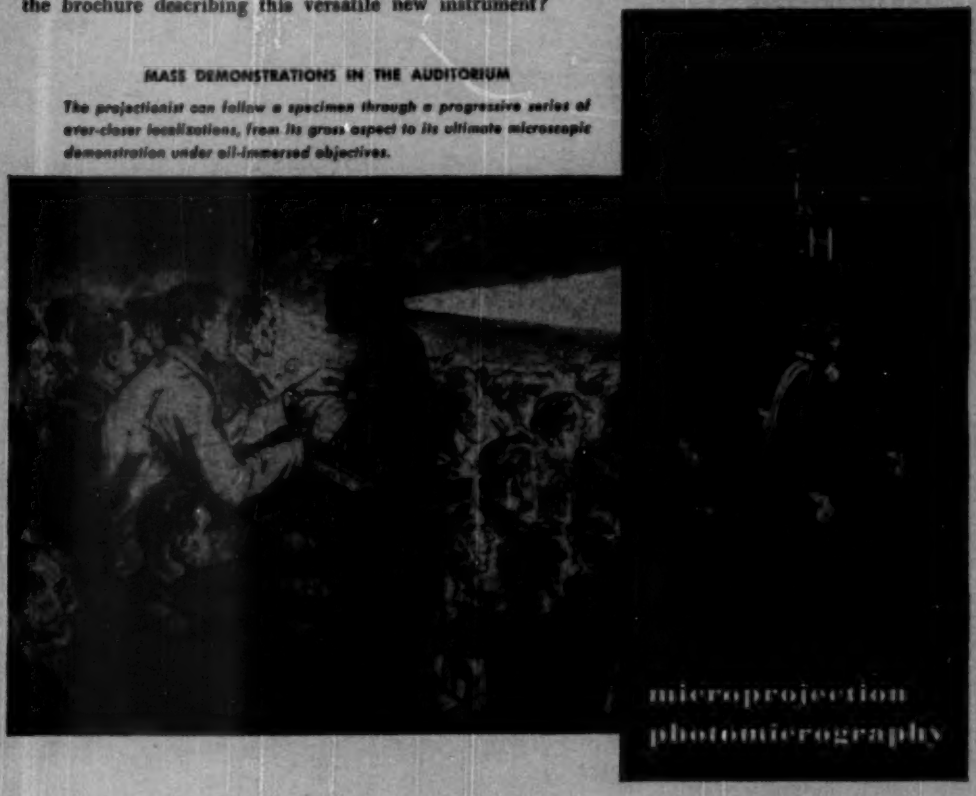
SMALL-GROUP STUDY IN A NORMALLY LIT ROOM

Image is cast in a dark-chamber table height screen: each observer has a separate light-excluding viewing hood.

SCOPICON, Inc.
215 East 149 Street
New York 51, N.Y.

MASS DEMONSTRATIONS IN THE AUDITORIUM

The projectionist can follow a specimen through a progressive series of ever-closer localizations, from its gross aspect to its ultimate microscopic demonstration under oil-immersed objectives.



microprojection
photomicrography